CIBA FOUNDATION COLLOQUIA
ON ENDOCRINOLOGY

Vol 13 Human Pituitary Hormones

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CIBA FOUNDATION COLLOQUIA ON ENDOCRINOLOGY

VOLUME 13
Human Pituitary Hormones

In honour of
PROFESSOR B A HOUSSAY, For. Mem R.S

Editors for the Ciba Foundation
G E W WOLSTENHOLME,
O B E M.A. M.B., M R.C.P
and
CECILIA M O'CONNOR, B.Sc

With 86 Illustrations



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The Ciba Foundation a unique international institution owes its inception to the generosity of CIBA Limited Basle However being established under British trust law it enjoys complete independence in practice and policy

Under the guidance of its distinguished Trustees the Foundation offers accommodation to scientists from all over the world at its home in Portland Place Foremost in its activities is the organization of small conferences the proceedings of which are published in book form in the manner of the present volume The Foundation convenes many other informal discussions between research workers of different disciplines and different nationalities and each year invites an outstanding authority to deliver a special lecture An exchange programme between French and British postgraduates is conducted and a library service is available Furthermore, the Ciba Foundation attempts in every other way possible to aid scientists whether they be Nobel Laureates or young graduates making their first original contribution to research

The purpose of the Ciba Foundation which is to promote international co operation in medical and chemical research is symbolized in the armorial bearings by five interlaced rings representing the continents a black sacrificial cock (emblem of Aesculapius) holding a medical caduceus and three regular hexagons for chemistry Its domicile in London is indicated by the red sword of St Paul and the British lion the wyvern and the crozier symbols associated with Basle refer to the sponsoring firm located in this ancient Swiss town

THE CIBA FOUNDATION

for the Promotion of International Co operation in Medical and Chemical Research

41 PORTLAND PLACE LONDON W 1

PREFACE

It was a suggestion by the late Professor Braun Menendez which led to the organization by the Ciba Foundation of a colloquium in Buenos Aires immediately before the 21st International Congress of Physiological Sciences During its preparation, an air crash caused the tragic death of Professor Braun Menendez, a scientist a colleague and a friend whose memory all will cherish with respect and affection

Professor B A Houssay whom the Ciba Foundation was privileged to honour by holding in his name this colloquium on Human Pituitary Hormones a subject owing so much to his own original and classical work generously spared time from the multitude of his responsibilities for the major Congress to obtain important governmental and local decisions which enabled the Foundation despite many difficulties to arrange its meeting

Dedicated and untiring assistance was given in Buenos Aires by Dr M R Malinow and much help also by Dr E Montuori in finding the path—there seemed to be no correct way—through the intricate maze of airline prices and reservations The colloquium was held in the City Hotel whose manager Mr R Papernik co operated most readily with Dr Malinow in meeting the detailed requirements of the Foundation

The Foundation s own team for this distant occasion consisted of the Director with his Scientific Assistant Cecilia O Connor and his Conference Assistant Nancy Spufford They received essential advice and encouragement in all the planning the journeys and the technical and social aspects of the colloquium from a Member of the Executive Council Professor F G Young In addition Professor Young s Chairmanship was a major factor in establishing the maturity and excitement of the scientific proceedings

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The papers, and the discussions they aroused are here reproduced The editors hope that these contributions from workers in eleven countries, assembled in the Argentine by a British Trust of Swiss origin, will provoke interest and stimulate further research in many parts of the world

CONTENTS

List of those participating in or attending the Colloquium on 'Human Pituitary Hormones' 6th–8th August 1959

EVELYN ANDERSON	National Insts of Health Bethesda Md
R A ATRIA	Hospital del Salvador Santiago de Chile
F A DE LA BALZE	Endocrine Inst National Inst of Health Buenos Aires
J C BECK	McGill University Clinic Royal Victoria Hospital Montreal
P M BOTTARI	Dept of Medicine and Pharmacology University of Brussels
E B DEL CASTILLO	Endocrinological Dept. Hospital Rivadavia Buenos Aires
J M CERVIÑO	Inst of Endocrinology Hospital Pasteur Montevideo
A B DE ULHOA CINTRA	Faculty of Medicine University of Sao Paulo
*E J CONWAY	Dept of Biochemistry and Pharmacology University College Dublin
G W CORNER	Rockefeller Inst for Med Res New York
A TACHELLO COSTA	Soc Argentina de Endocrinología y Metabolismo Buenos Aires
*V G FOGLIA	Inst of Physiology University of Buenos Aires
RUSSELL FRASER	Postgraduate Medical School London
C A GEMZELL	King Gustaf V Research Inst Stockholm
F GOMEZ MONT	Hospital de Enfermedades de la Nutricion Mexico D F
J I HARRIS	Dept of Biochemistry University of Cambridge
A B HOUSSAY	Centro de Endocrinología Buenos Aires
B A HOUSSAY	Inst de Biologia y Medicina Experimental Buenos Aires
C H LI	Hormone Research Laboratory University of California Berkeley
J A LORAINE	Clinical Endocrinology Research Unit (Medical Research Council) Edinburgh
R LUFT	Dept of Endocrinology and Metabolism Karolinska Hospital Stockholm
M R MALINOW	Inst of Physiology University of Buenos Aires
J MORATÓ MANARO	The Endocrinology Laboratory of the Inst of Endocrinology Ministry of Public Health Montevideo

* Unable to participate in the colloquium because of illness

O. H. PEARSON	Western Reserve University School of Medicine Inst. of Pathology Cleveland Ohio
M. S. RABEN	New England Center Hospital Boston Mass
C. H. READ	Dept of Pediatrics State University of Iowa Iowa City
R. R. RODRIGUEZ	Inst de Fisiología Universidad Nacional de la Plata La Plata
JANE RUSSELL	Dept of Biochemistry, Emory University Georgia
M. SONENBERG	Memorial Center for Cancer and Allied Diseases New York
ELEANOR VENNING	McGill University Clinic Royal Victoria Hospital Montreal
A. F. WILHELMI	Dept of Biochemistry Emory University Atlanta Georgia
F. G. YOUNG	Dept of Biochemistry University of Cambridge

CHAIRMAN'S OPENING REMARKS

F G YOUNG

IT is with great pleasure and indeed, with pride that I take the chair at a Ciba Foundation Colloquium held in honour of Prof Houssay in this great capital city of Argentina Perhaps the only claim that I have for appointment to this position is that I am a member of the Executive Council of the Foundation Furthermore I believe that I have known Prof Houssay and admired his work as long as or possibly longer than anybody in this room apart from those who live in Argentina Nobody will disagree with me when I say that Prof Houssay is one of the most distinguished citizens that Argentina has ever had and that he is the most outstanding scientist in South America Not only has he been responsible for much important original research about which I shall speak a little later but also he has been a rock standing firm in a sea of political trouble The tide comes the tide recedes but the rock stands as it always did It is with special pleasure that we all meet here today to honour one who has withstood so much and who has survived with dignity and calm

In 1950 I had the most interesting experience of lecturing in a number of countries in this vast subcontinent of South America Although I knew before how internationally distinguished Prof Houssay was I had not realized until then how, in all of South America he had also become a symbol of independent science In every country which I visited he was I found a scientific and a personal inspiration His activities have meant much more to the world than has been provided by the facts that his researches have elucidated vitally important though these facts have been

The poet Shakespeare who is known in this country as well as he is known in other parts of the world wrote 'The toad

ugly and venomous, wears yet a precious jewel in his head" That Shakespeare had the pituitary gland in mind when re ferring to the jewel in the head of the toad is unlikely but Prof Houssay's researches clearly established what a valuable jewel the hypophysis of the *toad really is, and how important* it is to have an experimental animal in which the different parts of the pituitary body can be separately removed with relatively little interference to the rest of the gland The jewel in the head of the toad has turned out to be a very valuable prize indeed

It is perhaps rather surprising now to recall that in the early nineteen twenties the metabolic importance of the pituitary gland as a whole was generally ascribed to the poster ior portion, the anterior lobe being considered by many as of much less significance I believe I am right in saying that it was the investigations of Prof Houssay and Dr Dora Potich with the toad in the late nineteen twenties that first revealed the important metabolic effects of removal of the pars glandu laris of the anterior pituitary gland especially on carbohydrate metabolism and experimental diabetes One disadvantage of the toad is that it wears its ' anterior ' pituitary lobe in a posterior position so that it is not correct to call the pars glandularis of its pituitary gland 'the anterior lobe In the hands of Prof Houssay and his colleagues the toad proved to be an immensely useful experimental animal and paved the way for the demonstration in mammals—the dog was impor tant in the later investigations—of what came to be known as the Houssay phenomenon , the alleviation of experimental diabetes by removal of the pituitary gland or of its anterior portion alone Subsequently Houssay and Biasotti as a corollary of the earlier findings showed that an extract of the anterior pituitary lobes of oxen exerted a diabetes inducing effect in partially depancreatized dogs This was in the early nineteen thirties I personally recall the scepticism with which the description of the Houssay phenomenon and of the diabetes inducing activity of anterior pituitary extracts was greeted at that time in some apparently authoritative

quarters A distinguished physiologist long since dead told me at that time that he thought these results must be wrong because the evidence from other laboratories showed that the posterior lobe of the pituitary was of predominant importance in the control of metabolic processes Nevertheless I was able myself to show that if one exactly repeated Prof Hous say s preparation of an extract of fresh ox anterior tissue administering this extract to a normal intact dog however one could demonstrate a clear diabetes inducing effect of this material

It would be dangerous to say of any scientist that nothing that he has published has ever been contradicted but I can say of Prof Houssay that nothing that I know about which he has published has ever turned out to be wrong People have failed sometimes to obtain the same results but in my experience their failure has usually been attributable to the fact that those attempting to repeat the experiments did not use precisely similar conditions

There is no doubt that Prof Houssay is one of the few responsible for the recognition of the importance that we now ascribe to the anterior pituitary gland Others whose names come to mind in this connexion are Drs Philip E Smith Herbert M Evans J B Collip and Hans Selye More recently the investigations of others including in particular those of Dr C H Li—coming as he does from the same laboratory as Philip Smith and Herbert Evans—have placed the identity and chemistry of certain anterior pituitary hormones on a firm basis

In reviewing the nature and mechanism of action of hor mones I recently pointed out that somewhat confusingly we are now less certain of the precise identity of some hormones than we were say ten years ago I raised the question of whether the substance that we ought to call a hormone is that which is present in the endocrine gland itself or in the blood flowing from the endocrine gland, or is it the substance which may be formed by metabolic changes in the blood stream or in non target organs from the material originally liberated by

the secretory tissues? Or is it whatever may ultimately appear as the result of metabolic changes in the tissues on which the hormone acts and which may itself be capable of evoking a biological response in the target tissues which the chemical substance in the endocrine gland or in the blood would not be capable of producing? Discussion of these questions appears to me not to be very useful at the present time, although the relevant experimental facts are, of course, of great importance When we come to discuss anterior pituitary growth hormone during this colloquium we shall surely find that this hormone provides an example of some of these complications Moreover, it seems possible that many hormones and the pituitary hormones in particular, are not the specific substances that we once thought they were and that a substantial overlap of properties may exist between different hormones and their relations Perhaps also no single target organ exists for many or all of them Indeed some of the rigid ideas of the past appear to be in need of modification We shall no doubt have further information on these points at the end of the present colloquium

There are five main stages in the development of experimental research concerning the endocrine activity of any particular organ or tissue Clinical observations and investigations often play a part in the early stages and sometimes in the later ones but the five experimental stages which I distinguish are as follows (i) the results of removing the endocrine organ or of damaging the secretory tissue in suitable experimental animals are first described (ii) crude extracts of the organ or of the tissue are then prepared and attempts are made by administration of these crude extracts to alleviate the symptoms which develop in the animal in which the organ or tissue is missing or damaged (iii) chemical isolation of active material is then effected and the chemical structure of the active principle or principles is elucidated (iv) the active substance or substances are produced in the laboratory by artificial means and the influence on biological activity of variations in chemical structure is assessed (v) the

mechanism of action of the substance or substances is then investigated and in these researches the activity of meta bolites and of chemical relations of the naturally occurring substances which may or may not naturally exist may be of particular importance Investigation of the mechanism of action of a hormone may run concurrently with all the four other phases but the results are most likely to be meaningful when pure preferably chemically synthetic preparations of the active hormones are available This is also true with respect to evidence that multiple activities may be ascribable to one hormone or that hormones may share activities in common It is reasonable to say that with respect to anterior pituitary hormones we are in stages (iii) (iv) and (v) although we can still argue quite strongly about stage (iii)—the chemical isolation of the pure substance and its chemical structure—with respect to some of these hormones at the present time

The production of peptide hormones in the laboratory by *artificial means is in its infancy But the infant is a lusty one* and is crowing loudly at the present time As a biochemist I am delighted that some at least of the discussions during this colloquium can be in terms of known chemical structures and moreover in some instances about chemical structures that can either be reached by synthesis already or will almost certainly be synthesized in the not far distant future On the other hand the mechanism of action of anterior pituitary hormones is still a matter of considerable doubt and discus sion I do not think that this is a subject on which there will be wide agreement during the present meeting

One striking point that has emerged from studies on peptide *hormones in recent years is the existence in some instances* of clearcut chemical differences between hormones prepared from different species of animal In his brilliant work on the structure of insulin Sanger clearly demonstrated that dif ferences exist in the structure of insulin from different species of animal although these differences were of a rela tively minor character and associated with the same small portion of the A chain of the insulin molecule Recent evidence

suggests that some hormones of the anterior pituitary gland exhibit much bigger species differences than have so far been found for insulin The growth hormone of the human pituitary gland differs very strikingly in chemical properties and structure from that of the ox pituitary gland This is a matter that will no doubt come up for consideration during this meeting Advantages may result from these species differences since one is sometimes able to produce antisera to certain hormones which is of great value for investigation and assay of the antigenic hormone This is an important development which we shall hear discussed during the present meeting

Another striking fact which has arisen in the course of recent investigations of the chemistry of pituitary hormones is the existence of a close chemical relationship between the melanocyte stimulating hormones of the pars intermedia and corticotropin This is another subject that will be considered in the course of our discussions, and I shall therefore say no more about the work of C H Li J I Harris and A Lerner in this connexion except to enquire perhaps, whether we might reasonably hope that some similar identities of sequences may exist between other pituitary hormones Certainly the possibility that some common building blocks may exist from which can be fashioned anterior pituitary hormones of different sorts according to different needs is a fascinating one

Species differences in anterior pituitary hormones may well account for the apparent lack of activity of the hormone from one species in another in certain instances The lack of growth stimulating action of ox growth hormone in the human being has been a mystifying and, indeed, a depressing fact, until relatively recently But the chemical differences now known to exist between the ox and the human hormones may well account for the facts as we know them at present The effect of human growth hormone in human beings will be a matter for fruitful discussion during our colloquium

When Prof Houssay and his colleagues were carrying out their careful investigations on the hormonal effects of removal

of the various parts of the toad pituitary gland in the nineteen twenties, an argumentative critic might have asked what possible value could this have for human medicine The fact that the removal of the pituitary gland is now practised as a therapeutic procedure in certain severe complications of diabetes would in itself be a complete and sufficient answer to that hypothetical question of more than thirty years ago But if further vindication is needed it is seen implicitly in the fact that we are meeting here in Argentina in honour of Prof Houssay to discuss the hormones of the human pituitary gland and their considerable clinical significance

Although man has not evolved biologically from the toad the colloquium that we begin today has clearly descended from *Bufo arenarum* which Prof Houssay and his colleagues employed so skilfully in the early days Some of you may have noticed that recently the Ciba Foundation has acquired a coat of arms Appropriately the College of Heralds in London agreed that this coat of arms which now appears on the Foundation s notepaper should include for the first time three benzene rings Benzene rings were certainly never thought of in early heraldry If Prof Houssay were to acquire a coat of arms surely it ought to include the parts of the pituitary gland gules with hypophysectomized toads as supporters

We all agree these days that international co operation is of vital importance to the future of the world We have here today a delightful example of international understanding The Ciba Foundation for the Promotion of International Co operation in Medical and Chemical Research was founded and is financed by a Swiss Company The Foundation has British trustees and a House in London It has arranged a conference in Argentina in honour of Prof Houssay attended by members of eleven different nations and the language of this conference in Argentina is English

Si bien el idioma oficial de la conferencia es el inglés es mi propósito decir en forma comprensible algunas palabras en

español Professor Houssay, me es grato testimoniarle nue stro agradecimiento por facilitar el motivo de nuestra visita a la Argentina Apreciamos sinceramente tener la oportunidad de reunirnos con Usted y con sus colegas en su proprio país y esperamos que Usted por su parte, encuentre suficiente mente interesante las discusiones del coloquio que se celebra en su honor

THE RADIOLOGICAL ANATOMY OF THE HUMAN PITUITARY

G F JOPLIN AND RUSSELL FRASER

Department of Medicine Postgraduate Medical School of London

CLINICAL and test evidence of pituitary hypofunction is always a little suspect without evidence of a cause this may be provided by a past history of postpartum haemorrhage but otherwise it is most often provided by signs of a tumour i e by radiological signs of enlargement of the sella turcica and similarly the clinical and test evidence of hyperpituitarism is very much more firmly based when there is radiological evidence of a tumour But again we cannot dismiss a clinical diagnosis of acromegaly without this support Cushing s syndrome without evidence of an adrenal tumour may also have its origin in a pituitary tumour, but even less frequently do we have radiological evidence to support this diagnosis Thus for many years one of the important diagnostic signs of pituitary disease has been the radiological demonstration of enlargement of the sella turcica Clearly this does not itself provide evidence of disordered pituitary function but as many of the tests of pituitary function are indirect it is always very important to supplement them by evidence of a disease process in the pituitary region Indeed gross enlargement and distortion of the pituitary fossa is sometimes an unexpected finding—metastatic tumours may cause enlargement often without any obvious symptoms or signs of pituitary dysfunction At other times patients being X rayed for suspected cerebral disease may be found to have an enlarged sella turcica and more careful examination and testing may then reveal hypopituitarism as an important factor in the patient s syndrome

How accurate is the radiologist in his assessment of the

limits of the range of the normal pituitary fossa dimensions? Gross enlargement is easily recognizable not only from the size of the sella, but also from its expansion into an unusual shape—most characteristically an expansion downwards and outwards into the sphenoidal sinus, considerable enlargement may also involve displacement of the clinoid processes and dorsum sellae Another useful sign is the appearance of two floors which provided they are not due to an untrueness of the lateral projection signify unequal expansion of one side of the sella A tomogram can clinch this interpretation and so the evidence of a tumour Less gross enlargement can usually be recognized not only by the size of the shadow of the sella turcica but usually also by some distortion of the floor due to its displacement downwards into the sphenoidal sinus At times the assessment of the sella turcica s size can throw some interesting light on the basis of a patient's syndrome, as illustrated by case J V (Joplin, Fraser and Harrison 1960) who presented as an apparently typical instance of exoph thalmic ophthalmoplegia (ophthalmic Graves' disease or severe exophthalmos without thyrotoxicosis) Here we have a sella which both by the criteria of lateral area, and of floor displacement down towards the sphenoidal sinus, must be classed as enlarged and so implying a tumour Does this case not offer important supporting evidence of the pituitary origin of this condition whether it be due to an excess produc tion of exophthalmos producing substance of thyrotropin or of a combination of the latter with adrenocorticotropin? But when the sella turcica does not show any such distortion in its shape, can we rely on an assessment of its area in the lateral view? In measuring the area of this lateral projection where should the upper limit be placed? And further if the radio logist could measure the sella width might not this be an important associated measurement? The measurements to be reported here offer some answers to these questions

Our special interest in the radiological anatomy of the pituitary really arose when we became involved in pituitary ablation by needle implantation of radioactive seeds into the

sella in cases of metastatic carcinoma (Fig 1) Rasmussen Harper and Kennedy (1953) had suggested using ^{90}Y for this purpose because of its highly localized necrotizing radiation from β rays of 2 3 MeV and Forrest and Peebles Brown (1955) first described a method of needle implantation Our early experience (Fraser *et al* 1959) established that such implantation could achieve functional hypopituitarism in at least 80 per cent of cases but also that we were not always confining the radiation to the pituitary gland—occasional partial oculomotor palsies being encountered and more seriously rhinorrhoea was a relatively frequent complication This latter complication meant that our radiation was damaging the diaphragma for only rarely is there any natural communication between the subarachnoid space and the pituitary fossa This experience demanded that we must check our landmarks for the diaphragma Indeed the conventional view that it lay at the radiological top of the sella seemed less likely than that it might dip downwards below this level The occasional damage to the oculomotor nerves suggested the need also for a careful check for the landmarks of the width of the gland Clearly we could only attain widespread pituitary destruction along with precise limiting of the damage to the pituitary if we had precise radiological landmarks for the limits of the gland tissue

For these reasons one of us (G F J) and our colleague Dr H J Bloch embarked on a postmortem study details of which have been published (Joplin and Bloch 1959) which was designed to ascertain the radiological landmarks for the precise limits of the pituitary gland and also for the important nerves in its neighbourhood This study aimed to define

(1) the landmarks for the diaphragma sella on the lateral skull film

(2) any bony landmarks which would indicate the width of the gland

(3) the position of the third cranial nerve in A P and lateral skull films

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Method of Study

At autopsy, after careful exposure involving the minimal displacement and a little careful dissection radio opaque clips and markers were placed on these structures, and then after careful replacement of the skull cap X rays were taken The sphenoid bone was then excised with the pituitary *in situ*, and further films were taken To help in defining the position of the diaphragma, radio opaque media were also injected into the sella, and also instilled via the cerebrospinal fluid on to the top of the sella with the brain *in situ* Finally after dissection the width of the gland was measured with dividers for correlation with the radiological measurements The 25° tilt fronto occipital view was found to offer the optimal A P projection since it outlined the dorsum sellae through the foramen magnum

Results

Landmarks

Fig 2 summarizes the results of these studies (i) the diaphragma was shown to extend from the tuberculum sellae to an attachment on to the anterior limit of the posterior clinoids (which was usually their closest point to the tuberculum sellae) In no instance was the attachment found to the top of the posterior clinoids as has often been stated to be the case indeed in some cases a free spike was found to project several mm upwards from one or both clinoids above the level of the diaphragma (ii) The width of the gland measured after dissection was found to correspond with the width of the waist of the dorsum sellae as seen in the 25 tilt fronto occipital view when corrected for magnification All but one pair



Fig 1 Implantation into the pituitary by needle

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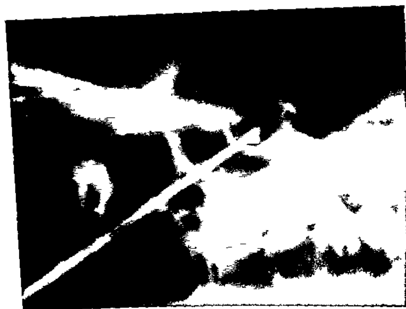
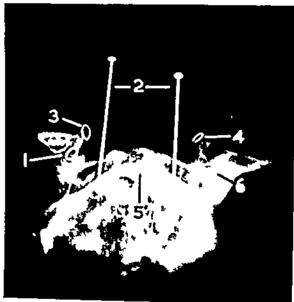
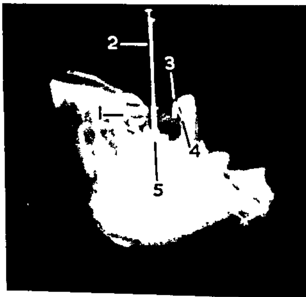


Fig 1 Implantation into the pituitary by needle



3



3b

occipital views of pituitary area in excised sphenoid (previous ablation with per surface of the gland is visible as a soft tissue shadow

l nerves at level of tuberculum sellae

l nerves at level of perforation of the dura

ateral margins of gland

5 the · s sec ls

(From Joplin and Bloch 19 9 Reproduced by permi sion of the Editor *Brit J Ra hol*)

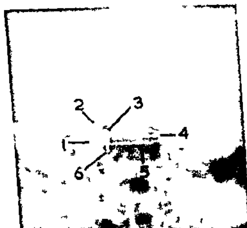
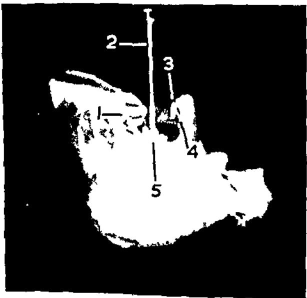


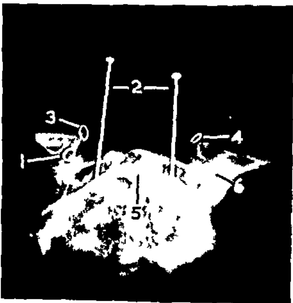
Fig 3c A 25 tilt fronto-occipital radiograph of pituitary area in cadaver

1 and 2 clips on right 3rd nerve
3 and 4 clips on posterior clinoids
5 transverse pin along lower border of optic chiasma
6 clip on the right lateral margin of diaphragma sellae

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3a



3b

Fig. 3a and b Lateral and tilt fronto occipital views of pituitary area in excised phenoid (previous ablation with radioactive seeds) *The upper surface of the gland is visible as a soft tissue shadow*

1 and 6 rings on 3rd nerves at level of tuberculum sellae
3 and 4 rings on 3rd nerves at level of perforation of the dura
2 *pins at lateral margins of gland*
5 the seeds

(From Joplin and Bloch 19 9 Reproduced by permi ion of the Editors *Brit J Ra hol*)

of these measurements agreed within 1 mm and no correlation was found between the gland width and the distance between either the anterior or posterior clinoids (iii) The third nerve was found on the two views (*a*) on the lateral lying parallel to the diaphragma at the level of the undersurface of the anterior clinoids and (*b*) on the 25° fronto occipital view lying on a line running from the lateral convexity of the posterior clinoids to the tip of the anterior clinoids In no case

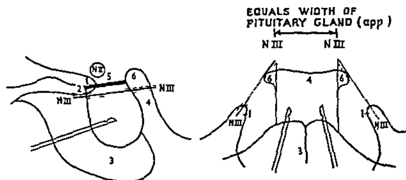


FIG 2 Diagram of relations of pituitary gland as demonstrated radiologically The needle for implanting ^{90}Y seeds is shown *in situ*
Left lateral view
Right 25 fronto occipital view
(From Fraser *et al* 1959 Reproduced by permission of the Editors *Lancet*)

did the nerve deviate more than 2 mm from this line Some of the X rays leading to these conclusions are illustrated in Fig 3*a* *b* and *c*

Dimensions of the normal adult pituitary fossa

In the literature there are available several reports of the dimensions of the normal pituitary fossa but none states both the magnification of the X ray apparatus used and the exact limits used for defining the fossa (Table I) Our X rays taken by the Lysholm skull table apparatus with a film focus distance of 28 in were found to require a constant magnification

correction i e to give the true sella measurements, the lateral film required reduction to $\frac{5}{6}$ and the 25° fronto occipital projection required reduction to $\frac{4}{5}$ Since the various diameters proposed in the past have not been related either to easily identified landmarks or to the exact pituitary depth we have suggested the following procedure which can be clearly followed on any skull film and also measures true gland

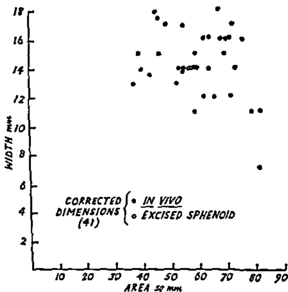


FIG 4 Relation between area in lateral view and width of human sella turcica (corrected for magnification)

dimensions as may be seen in Fig 1 First the nasion tuberculum line is found The greatest fossa length parallel to it is measured and then the greatest depth perpendicular to it measuring from the diaphragma to fossa floor Finally the cross sectional area below the diaphragma is easily measured using a mm grid etched on perspex In addition the width may be measured from the waist of the dorsum sellae

Table I

NORMAL PITUITARY FOSSA MEAN (RANGE) IN MM

	Width	*Depth*	*Length*	*Area*	*Basis*
Jorlin and Bloch (1959)	15 (12–2.)	8 (7–10)	11 (8–14)	87 (.0–118)	50 fem adult*
Pendergrass Schaeffer and Hodes (19 0)		8 5 (–10)	9 5 (–12)	—	10 per cent magnification
Camp (19?1)		(5–16)	(4–12)	—	500 male + fem
Hare Silvens and Smedal (1940)				(–130)	700 male + fem
Mahmoud (1958)				80 (22–126)	100 male + fem *

Lysholm table

Summary

1 Certain new radiological landmarks of the human pituitary have been defined

(a) The diaphragma passes from the tuberculum sellae to the closest limit of the posterior clinoid This defines how near the optic nerve may come to the pituitary
(b) The width of the pituitary approximates to that of the waist of the dorsum sellae
(c) The third nerve passes a little further laterally as defined in the text

2 The size of the normal pituitary fossa may be best indexed from its area in the lateral view whose normal limits have been defined

REFERENCES

CAMP J D (1924) *Amer J Roentgenol* 12 143
FORREST A P M and PEEBLES BROWN D A (1955) *Lancet* i 1054
FRASER R JOPLIN G F LAWS J W MORRISON R and STEINER R E (1959) *Lancet* i 382
HARE H F SILVENS E and SMEDAL M I (1949) *Radiology* 52 193
JOPLIN G F and BLOCH H J (1959) *Brit J Radiol* 32, 380 527
JOPLIN G F FRASER R and HARRISON M (1960) *Proc roy Soc Med* in press
MAHMOUD M E S (1958) *Brit J Radiol* suppl 8 p 6
PENDERGRASS E P SCHAEFFER J P and HODES P J (1956) *In* The Head and Neck in Roentgen Diagnosis vol 2 p 928 Oxford Blackwell Scientific Publications
RASMUSSEN T B HARPER P V and KENNEDY T T (1953) *Surg Forum* 4 681
SCHAEFFER J P (1924) *Anat Rec* 144 391

DISCUSSION

Young With regard to your observations on the sella turcica can you place any limits to the normal? I am not thinking of the shape but of the area What do you regard as abnormal in terms of a mean value and its standard deviation?

Russell Fraser Our figures were based merely on the measurements of 50 individuals

Young Can you give any statistical information about them?

Table I gives the limits, uncorrected for magnification of the normal range so defined, with which in general the ranges previously defined agree Previously depth has been more variable perhaps because of the uncertainty of the position of the diaphragma, and width has not been available There is evidently more variation in area or volume than in any single diameter, which was perhaps a little surprising In order to examine this point further we assessed the correlation between width and the lateral area It will be seen from Fig 4 that there is no evidence of any inverse correlation between these dimensions We may conclude from this that the area measured in the lateral view gives an adequate and perhaps the best index of the size of the fossa there does not seem to be any tendency for narrow glands to have the larger lateral areas

Application of the landmarks

We were also now able to go over the X rays of our previous implants where placement had not been so satisfactory and to study the incidence of the complications in relation to the distance of the seeds from the diaphragma or of the third nerve This showed that seeds placed beyond 4 mm from the third nerve location did not cause paresis and that no rhinorrhoea followed when no seeds were placed within 3 mm of the diaphragma It may be added that each of the seeds used has a necrotic radius of 3 mm (delivering above 100 000 rads with in that range but this may vary slightly with the hotness of the seed and also with the proximity of other seeds) This seemed further evidence for the validity of our anatomical conclusions Since completing these studies, we have been able to make our implantations in relation to this anatomical information keeping the necrotic radius of the seeds just within the definable limits of the gland and avoiding the danger areas, fortunately this has eliminated our oculomotor palsies and practically eliminated our incidence of post operative rhinorrhoea

look at the sella turcica from above in a skull these are the obvious places where you would expect it to be attached The clinoids are obviously for a ligament to go between them and the rim with a little edge on it is seen on the anterior surface where the tuberculum sella is presumably where you would expect the fascia to be attached

Rodriguez Is there any sex difference in your normal values ?

Russell Fraser Our normal values were all obtained from females since we felt that they should not be mixed We have yet to examine a set of males I suspect that there will be a difference

de la Balze Are there any differences with age ?

Russell Fraser These were only adults I do not think that we saw any evidence of a correlation with age within the adult range Of course in the younger and immature subject it must be related to age

Loraine In the patient with exophthalmos with a very large sella turcica was there any evidence of abnormal thyroid function ?

Russell Fraser This particular patient by the time he reached us had been taking a preparation for enteritis which has a lot of iodine in it therefore we were unable to measure his thyroid function with ^{131}I but it has been measured previously and found to be quite normal We found his basal metabolic rate to be normal and I have every reason to believe that his thyroid function was normal or at least not measurably abnormal

de la Balze Prof Russell Fraser how frequently does diabetes insipidus occur ?

Russell Fraser When we first placed the needles in the pituitary we aimed to destroy the anterior two thirds but of course the posterior wings of the anterior pituitary come well back and lately we have placed more needles posteriorly Even in our earlier cases there was a considerable oedema following irradiation so that the immediate effect in most cases was diabetes insipidus for perhaps one to three months After three months the diabetes insipidus has usually disappeared completely We assumed this was because the damage was confined to the infratentorial tissues and there was enough posterior pituitary tissue above the tentorium to avoid diabetes insipidus I think the surgeon usually has a slightly different experience but he must inevitably touch the supratentorial tissue

Beck In the case where you get ballooning of the sella turcica does that allow the placement of yttrium needles so that you can still get the portion of the tumour which is bulging and avoid radiating the optic nerve or the third and the sixth nerves ?

Russell Fraser That calls for precise placement Fortunately when we deal with these tumours we usually put radioactive gold

Russell Fraser Those were the absolute limits and I think they exceed 2 S E on that sample of 50 They are to be defined as normal limits in that sample of 50 individuals I think that we should have to have a wider range of anatomical sampling before we could be as accurate as we would like to be but I doubt whether they would change very much from that

Young Do you feel confident about the reliability of your figures as being applicable to a wider sample ?

Russell Fraser Yes, I think so They are based on an adult female population obviously those of males would require studies which we have not yet made and also to define those of the young would require a further study

Malinow Are you familiar with any studies on the increase of pressure within the gland that is necessary to increase the size of the bony parts around the gland ?

Russell Fraser No I am not

Malinow It would be interesting to know the relationship between that and the venous pressure within the gland for instance

Russell Fraser Perhaps we should measure it

Laqi Prof Russell Fraser I wonder whether you have to take into consideration the size of the skull and perhaps use that as a parameter ? Have you found any correlation between the size of the skull measured by different diameters and that of the sella turcica ?

Russell Fraser I did not mention the point that when we examined these normal measurements on the 50 subjects, unfortunately small in number we attempted to correlate the size of the skull as measured either by an anterior posterior diameter or a lateral diameter There did not seem to be any correlation between the size of the skull and the dimensions of the sella turcica I would suspect that some factor of a different order is involved in this variation It may be more related to differences in body build rather than to the absolute size of the skull

Corner When the diaphragma is pushed up by excessive growth of the gland does it retain its original attachments and merely bulge in the centre ?

Russell Fraser We have been anxious to define this in subjects with acromegaly or other tumours in the pituitary and have done so with the insertion of air in the cerebrospinal fluid followed by X rays It is difficult to interpret some of these air pictures because there is a dome in the middle with air coming down on either side There is no doubt that it retains its fixation at these points we showed but it may bulge up quite a bit Only after considerable enlargement is the whole dorsum sellae pushed away In fact, if you

an exit at the side so that we end up with two or more cones of seeds which can be anterior and posterior on each side We believe that if we put the seeds where we wish and if we read the anatomy correctly it is very feasible to destroy completely the pituitary by a pattern bombing of this sort A very considerable advantage of this procedure as against surgery is that while we pattern it to have the whole pituitary covered at 100 000 rads and upwards the few remaining bits that we may leave outside the pattern will have at the very least 10 000 rads so that these little remnants that we may leave are very sick remnants both from the radiations they received and from the interference with the blood supply A possible advantage over surgery is that the likelihood of regrowth of tissue left behind is very much less In our experience hitherto there has been no single instance where there has been any recovery of the impaired pituitary function We have of course had patients who had incomplete hypophysectomies where our placement has been imperfect But where we have obtained satisfactory test evidence that pituitary function was lowered at the three month postimplant tests, we have never found any evidence of return of function up to two or three years later This we believe is the important point It may be unimportant whether we destroy 90 or 99 per cent but it is important that we destroy enough to prevent regrowth and return of function On that criterion we believe that the radiation procedure has many advantages over surgery

We have made a few anatomical studies of our patients pituitaries We do not completely destroy the pituitary we nearly always leave a little rim of varying extent In the earlier days it was liable to be on the posterior extensions around the side Now we put the seeds fore and aft to a wider extent and hope that that will be catered for If we just exclude those areas many of the cells remaining in the peripheral rims are very sick looking

As regards the test evidence being sufficient it seems to us that if you have clinical or physiological tests showing very strikingly lowered function equivalent to that after total destruction that is the objective rather than an anatomical objective

Wilhelmi Do your tests show that the functions drop out in any particular order?

Russell Fraser In the early days we used to wait and see how quickly the result appeared but sometimes it would be at 2 a m in the morning and sometimes at 2 p m in the afternoon It was an unpredictable time of crisis which was very unsatisfactory Now we routinely give the patient cortisone for ten days and then withdraw it to see what function remains This we do in order to have a planned time for looking for signs of deficiency In any case this is

with a lower level of radiation of the order of 10–30 000 rads as our objective rather than full destruction In these circumstances we set ourselves the upper limit of 10 000 rads at where we place the diaphragma because we believe that this is the limit that the optic nerve will stand For this purpose we can take account of the bulge and so far we have not had any trouble with the optic nerve in these tumour cases

Bell Would you comment on the physiological evidence or the criteria which you use for total destruction using yttrium needles?

Russell Fraser Primarily we like to have evidence of lower thyroid function—as evidenced by ^{131}I—and associated with it evidence of cortisol deficiency which is shown by the need for cortisone to avoid serious symptoms by water diuresis test abnormalities and by the electrocardiogram We find the electrocardiogram is perhaps one of the most convenient methods After having obtained these two types of evidence which are readily obtained in all subjects (and *without enormous laboratory trouble*) *we pass to the hormonal* assays and measure the gonadotropins and steroids to check these indications of the first tests In general steroid assays have proved rather unhelpful in our experience because so often the patient is very ill beforehand and then they are abnormal in any case We feel that the important evidence is that of thyroid function and adrenocortical production of cortisol

Pearson Prof Russell Fraser do you feel that you are accomplishing total hypophysectomy by this means? Have you examined any of the sella turcica by serial sections afterwards? Although we have not had any personal experience with this approach it seems to me, on theoretical grounds that to attempt the total destruction of the pituitary by this means is a Herculean task I assume that you have to put seeds all through the pituitary within a range of 3 mm and it seems to me that it is almost impossible to accomplish a total hypophysectomy by this means With regard to the function test e g disappearance of gonadotropins suppression of thyroid and adrenal function from our experience with surgical hypophysectomy we feel that they are quite reliable criteria but not completely reliable for total hypophysectomy With the surgical procedure we sometimes find on serial section very small microscopic foci of anterior pituitary tissue left which in occasional instances may represent a fair percentage of the total gland Even with the surgical approach and cauterization it is sometimes quite difficult to obtain total removal of the gland

Russell Fraser With regard to the anatomical possibility of placing the seeds, our insertion procedure is done by two needle insertions, one on the left and one on the right side The needle has

whose carcinoma was complicated by a middle cerebral arterial thrombosis She died due to a combination of amentia hemiparesis and residual cerebral infection Nowadays we can usually control the infection by rapid institution of antibiotic treatment In this patient due to difficulty in interpreting the signs because of hemi paresis we withdrew the antibiotics too early and when they were reinstituted they were not as effective

As regards morbidity for instance in our 1959 cases where we know the anatomy a little better we had no occular paresis and an incidence of 12 per cent rhinorrhoea which I am happy to say we were able to stop in three instances

de la Balze If you did not get hypopituitary symptoms and signs the first time did you repeat the placement of seeds ?

Russell Fraser Yes always in cases where the implant does not produce a functional hypopituitarism we do a second implant and occasionally we have done a third implant We have one patient whose sella turcica is almost jammed full of yttrium we hardly see a niche left for another seed but still she has not hypopituitarism according to our tests She has had a magnificent clinical response in her carcinoma lasting two or three years with that We wonder if she may have a little pituitary tissue in the roof of the nose She is the only patient in whom continuation of the procedure has not been a practical proposition for full destruction

Gómez Mont How often do you have to make a second implant ?

Russell Fraser We obtain about 80 per cent of hypopituitarism on first implant according to our test

Luft The question of which method of hypophysectomy is to be preferred cannot be answered categorically It is influenced by the kind of disease you are going to treat and by the staff of surgeons available in your hospital It has been our experience at least that a good neurosurgeon will perform the operation with very little harm to the patient even if the condition of the patient before the operation is very bad So if you have a good neurosurgeon in your hospital it might be best to use the procedure of transcranial ap proach On the other hand a number of other procedures for re moval or destruction of the hypophysis have been suggested which may be taken as a sign of the need for non neurosurgical procedures in some places In my country the transantro sphenoidal approach of Hamberger Norlén is now widely used by the ear nose and throat surgeons it has the advantage of being extracranial This procedure may be preferable also in cases of diabetes mellitus with vascular complications as well as in patients with enlargement of the sella turcica due to pituitary tumours If you have not the facilities for the above procedures you may use pituitary destruction by

so quick that it would be difficult I suspect to see much difference Adrenal crisis used to develop between the fourth and eighth day

Beck Had the urinary gonadotropins disappeared at that time?

Russell Fraser We have not tested them at that time we have usually done only the adrenal and the thyroid function tests then and later the urinary gonadotropins

de la Balze Where do you put the seeds in the case of an acromegalic with eosinophilic tumour?

Russell Fraser As shown in my slide of an acromegalic tumour (Fig 1) one seed was placed anteriorly in the lower left hand part and the other posteriorly in the right hand part of the pituitary so that there is an even dose distribution through the pituitary with the minimum on the roof the optic nerve is the limiting factor there Most of the gland gets 15–30 000 rads but the rims get 10 000 rads

Luft It is well known that some degree of decrease in adrenal function may occur even in intact people, when you suddenly stop an administration of cortisone that has been of some duration Now these patients of yours have been on 50 mg of cortisone and more daily for ten days after hypophysectomy *If ten days* after hypophysectomy you have ten per cent of pituitary tissue left and you then withdraw cortisone don t you think that any such *patient would show signs of adrenal cortical insufficiency?* Perhaps even 20 per cent of pituitary tissue would not be enough to prevent a crisis

Russell Fraser I wish they all went into crisis! Unfortunately, we do not get 100 per cent success in our anatomical placement and there is a very striking difference between the patient who will show test evidence of hypopituitarism and the patient who will not I do not believe that ten days on 50 mg is enough to suppress seriously the adrenal cortex Perhaps others will have different views In fact about 20–30 per cent of our patients do not show evidence of hypopituitarism on the first implant When we withdraw cortisone they develop no symptoms With these patients then we have the opportunity to go in again as in ^{131}I treatment of thyrotoxicosis and we do put a few more seeds where gaps appear on the X ray of the skull We are usually happy to see cortisone dependence appear after the second implant

Pearson What are the overall mortality and morbidity rates in your series of cases?

Russell Fraser Our overall mortality is one patient in a total of 100 Of course many of these patients will ultimately die of carcinoma But the mortality within ten days of the implant or later attributable to the implant consisted of one patient who got meningitis from rhinorrhoea very early after the implant and also

SYSTEMATIC FRACTIONATION OF HUMAN PITUITARIES*

ALFRED E WILHELMI

Department of Biochemistry Emory University Atlanta Georgia

THE isolation of pure bovine growth hormone by Li, Evans and Simpson (1945) and the subsequent improvements in methods of preparation (Wilhelmi Fishman and Russell 1948 Li 1955 Raben and Westermeyer 1951) stimulated many studies of the effects of this hormone in experimental animals and in man It was both disappointing and of great interest to learn that in man and in monkeys as well purified bovine growth hormone and the somewhat less pure preparation from pig glands were totally ineffective (Shorr *et al* 1955 Knobil and Greep 1959 see also Russell and Wilhelmi 1958) The isolation and partial characterization of purified growth hormone from human and monkey pituitaries (Li and Papkoff 1956) revealed that the growth hormones of man and monkey differ significantly from bovine growth hormone and provided a physical and chemical basis for their differences in specificity The demonstration that both monkey and human growth hormone are highly effective in man (Beck *et al* 1957 Raben 1959) and in the monkey (Knobil *et al* 1957 Knobil and Greep 1959) has awakened an active interest in the hormones of the human pituitary This colloquium is an expression of that interest It affords us the privilege of an intimate discussion of the problems presented by the hormones of the human pituitary as well as the special privilege of enjoying our discussion in the hospitable country and the honoured presence of Professor Houssay, who can as an early pioneer

* This work has been generously supported by Grant C–2292 from the National Institutes of Health

seeds of radioactive yttrium This procedure has been used by Dr Notter in our place for some years he is using the procedure of Forrest with some minor modifications

Russell Fraser Dr Luft, it was because of the lack of a good neurosurgeon that we started out on this procedure, with such non experts as physicians and radiologists being the operators! We felt that as long as we did not go beyond the needle and avoided the knife then it was within the province of their position Another consideration was of course the fact that these patients were very ill I am sure that a very large proportion of our patients could never have been operated on by an ear nose and throat surgeon or a neurosurgeon The very ill patients included some who had hypercalcaemia and a blood urea of 300 and so could not stand an operation We were receiving the bones thrown from the table of the surgical masters when we started this procedure and many of our patients were very ill

Malinow Do you think your procedure could be used to destroy only the posterior pituitary gland? Sometimes we have very ill cardiac patients who are water logged and we have been wondering if destruction of the posterior pituitary gland would help them

Russell Fraser That is a very interesting point Nobody has asked me where is the dividing line between the anterior and posterior pituitary in our anatomical presentation This is one of the questions we still have ahead of us we have felt it is important to find that out accurately It is of course a curve and not a straight line In the standard text books we are told that it is the anterior two thirds and the posterior one third and I think that is approximately true It should be probably quite practical to do with the posterior what we do with the anterior i e to aim to destroy all the anterior and whatever amount of the posterior is necessary to ensure this If one applies that in converse one could aim at completely necrosing the posterior one third of the sella turcica and incidentally burn away some of the lateral arms of the anterior or pars glandularis I think one could destroy it within those limits

Sonenberg In order to complete Dr Luft s list of possibilities and in answer to Dr Malinow we could mention with regard to the posterior pituitary that perhaps one of the so called analogues of the du Vigneaud group or the Swiss group would be equally appropriate e g isoglutamine and acetyl derivatives as well as some monopeptides seem to have competitive inhibitory activity to the posterior pituitary principles

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* This work has been generously supported by Grant C-229_ from the National Institutes of Health

and a seasoned and still vigorous frontiersman in this field, lay sound claim to being an inspiration of much of the work to be discussed

In the past, the isolation of single pituitary hormones has been achieved from the glands of animals—ox, pig, sheep—that are both numerous and in easy commerce Methods have tended to be specialized for a single hormone or perhaps two since there was no reason for economy of raw material Only a few attempts at devising a general system for the separation of all the active principles of the anterior pituitary have been made (Fevold *et al*, 1940, Bonsnes and White, 1940, Ellis, 1958) and these have not been entirely satisfactory Our earlier work with fish pituitaries (Wilhelmi 1955) and more recently with monkey and human pituitaries has made it seem worth while to return to the problem of devising a general method in order to make possible the most effective use and application of these very scarce raw materials This paper describes some of our efforts to devise procedures by which all of the active principles of the human anterior pituitary might be separated and purified or concentrated A long step toward this desired goal can be taken if one can achieve some separation and concentration of the hormones by a process of selective extraction Once this has been done the further separation of individual hormones from the extracts can, in most cases be carried out with relative ease The work to be described has not it may be said at once arrived at a solution of the general problem It does however throw an interesting light on some peculiar characteristics of the human pituitary, and it gives promise (on an optimist's estimate) that the general problem can be solved

Sources and preparation of materials

Human pituitaries have been collected at autopsy and preserved under acetone in four Atlanta hospitals In addition a number of samples of acetone dried pituitaries have been donated by investigators interested in obtaining growth hormone Two lots of fresh frozen pituitaries have been col

lected in New York hospitals and sent to this laboratory for processing

The pituitaries stored under acetone were freed of the solvent by filtration and brief drying in an air stream on the filter They were then cut up into small bits and the particles were soaked in water and then lyophilized The material dried in this way powders easily in a mortar, and the dry powder is readily wetted by the aqueous solvents used in the extractions

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Methods of assay

Growth activity was measured by the gain in weight of 100 g male hypophysectomized rats 10–14 days after operation during 10 daily injections of the fractions under test (Marx Simpson and Evans 1942) Two doses of standard and of unknown were used in each case and the potency of the unknowns was estimated from the parallel dose response curves The standard was a purified bovine growth hormone GH_B168 prepared in this laboratory and equipotent with the U S P Standard A unit is defined as the activity of a milligram of the standard

Prolactin activity was measured by the increase in the weight of the crop sac of 6 week old White Carneau pigeons during four daily intramuscular injections of the substance under test (Riddle, Bates and Dykshorn 1933) For the screening of crude fractions, a single total dose of 10 mg was

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used In more refined assays, two doses of standard and unknown were employed and potencies were estimated from the parallel dose response curves The standard was a purified ovine prolactin (NIH SP 1) estimated to contain 15 U S P units per mg

Follicle stimulating hormone (FSH) was measured by the method of Steelman and Pohley (1953) using 25 day old Sprague Dawley rats Relative potencies were estimated from the parallel dose response curves of standard and unknowns The standard was an Armour FSH preparation 264 151 X A unit is defined as the activity of a milligram of the standard

Luteinizing hormone (LH) was assayed by the ovarian hyperaemia method of Ellis and Porter (1957) In this procedure the plasma of immature female rats is labelled by an intravenous injection of ^{131}I labelled serum albumin, and the degree of ovarian hyperaemia induced one hour after the intravenous injection of a preparation containing LH is measured by the amount of radioactivity found in the ovaries Estimates of relative potency were derived from the parallel dose response curves of standard and unknowns The standard was an Armour LH preparation 227 80 A unit is defined as the activity of 1 mg of the standard

Thyroid stimulating hormone (TSH) was assayed by the method of Lamberg (1953) using the ^{3} P uptake of the thyroids of 2 day old White Leghorn chicks Relative potencies were calculated from the parallel dose reponse curves of the unknown preparations and the U S P Thyrotropin Reference Standard

Adrenocorticotropic hormone (ACTH) was estimated by the adrenal ascorbic acid method of Sayers, Sayers and Woodbury (1948) The reference standard was the International Standard estimated to contain 1 unit per mg

Procedures and results of general extraction methods

Since the method of Wilhelmi Fishman and Russell (1948) and its later modification (Wilhelmi, 1955) had been used

successfully in the preparation of growth hormone from the pituitaries of a number of species of animals an attempt was made first to apply this procedure to human pituitaries The glands were subjected to two successive extractions The first extraction was conducted overnight in 0 3 M KCl at pH 5 5 The gland residue was then extracted overnight, at room temperature in 0 1 N HCl The choice of these conditions was based on the following considerations From the behaviour of ACTH and prolactin in a variety of conditions of extraction it was expected that these substances would remain out of solution at pH 5 5 in the presence of a moderate concentration of salt The primary extract should therefore contain growth hormone LH TSH and FSH Acid extraction of the gland residue should bring out the ACTH and prolactin As will be seen these expectations were only partly fulfilled

The primary extract yields five fractions

(A) 10 per cent ethanol pH 8 5
(B) 30 per cent ethanol pH 7 5
(C) 30 per cent ethanol pH 5 5
(D) 30 per cent ethanol pH 4 4
(E) 30 per cent ethanol 5 per cent trichloroacetic acid

The acid extract treated by a modification of the procedure of Li (1952) yields two fractions

(F) pH 3 12 per cent saturation with sodium chloride
(G) pH 3 96 per cent saturation with sodium chloride

The first of these is expected to contain prolactin the second ACTH Several extractions were made with this procedure Table I presents representative data from one such experiment At this stage of the work the activity most sought after was growth hormone and the assays of the fractions are most complete in this respect

It will be seen that in the primary extract growth promoting activity is distributed over fractions A–D that it is not suitably concentrated in fractions B and C as is the case with bovine pituitaries and that the total measured activity falls

Table I

YIELDS AND GROWTH HORMONE CONTENT OF FRACTIONS FROM HUMAN PITUITARIES EXTRACTED
(1) WITH 0.3 M KCL pH 5.5 AND (2) 0.1 N HCL pH 1.3
(Starting material 24.1 g acetone dried glands 120 g equivalent fresh weight)

Fraction	Weight mg	Weight g/kg	Growth activity units/mg	Growth activity units/kg
A	281	2.34	0.25	580
B	673	5.61	0.18	1 010
C	554	4.62	0.45	2 080
D	654	5.45	0.19	1 040
E	321	2.68	—	—
Subtotal	2 483	20.70		4 710
F	2 000	16.67	0.42	7 000
G	827	6.89	—	—
Total	5 310	44.26		11 710

(The weight reference is to fresh gland weight.)

far short of that reported by other workers (Li and Papkoff, 1956, Raben, 1957, Lewis and Brink, 1958). The report of Lewis and Brink that the growth promoting activity is only partially extracted under the conditions used here, and that as much or more activity could be extracted from the gland residue in 0.1 M KCl at pH 11, led us to examine the presumed 'prolactin' fraction F from the acid extract. This large fraction was highly active accounting for more than half of the total growth promoting activity of both extracts. The extraction with 0.3 M KCl at pH 5.5 therefore leaves behind a major portion of the growth hormone in the glands. This proved to be the case both with fresh and with acetone dried material. It is remarkable that human growth hormone can survive exposure to pH 1.3 for 24 hours at room temperature. It is also clear that this procedure does not lead to the desired concentration of the hormone in only one or two fractions or to its clean separation from other activities likely to be present in some of the fractions.

The recovery of growth hormone from fractions B and C was attempted by applying the procedure described by Wilhelmi (1955) for final purification of bovine growth hormone The results are tabulated in Table II With fraction B there was a large residue insoluble in 0 1 M KCl at pH 11 With both fractions the precipitation from 25 per cent ethanol at pH 7 4 was incomplete and the cloudy supernatant solutions could be clarified only by adjusting the pH to 5 5 (fraction C, Table II) Although a very active fraction was obtained from

Table II

RECOVERY OF GROWTH HORMONE FROM FRACTIONS B AND C

Fraction B (660 *mg* 119 *units*)			
Fraction	*Weight (mg)*	*Units/mg*	*Total units*
R	205	—	—
A	113	0 21	24
B	46	0 42	19
C	33	0 28	9
D	34	0 15	5
Total	431		57
Fraction C (540 *mg* 243 *units*)			
A	128	0 2[illegible]	28
B	157	1 10	173
C	15	—	—
Total	300		201

Initial concentration 1 per cent in 0 1 M KCl pH 11 R insoluble in 0 1 M KCl pH 11 A precipitate from 0 1 M KCl pH 4 5 B precipitate from 25 per cent ethanol pH 7 4 C precipitate from 25 per cent ethanol pH 5 5 D final supernatant solution dialysed lyophilized

fraction C the recovery of activity was poor especially from fraction B

The purification of growth hormone from fraction F (Table I) was achieved in two stages The fraction was extracted with 0 1 M ammonium sulphate at pH 7 and active material was precipitated from the extract by raising the concentration of ammonium sulphate to 2 M A third fraction was recovered

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(1) WITH 0.3 M KCL PH 5.5 AND (2) 0.1 N HCL, PH 1.8

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weights 46 000 27 000 or 1 7 1 An assumption implicit in this calculation is that all other factors in the assay are equal

The disappointing behaviour of growth hormone in this method of extraction was so distracting that not much effort was made to examine the fractions for other activities Dr H B Van Dyke has explored fractions A D and E for their content of gonadotropin, and has found good activity (mainly LH) in fractions D and E He and his colleagues will report on this work elsewhere

Much the same results were obtained in a single trial of the method of Ellis (1958) involving extraction of the glands with 0 1 M ammonium sulphate at pH 5 5 and subsequent fractionation of the extract with ammonium sulphate at pH 7 and pH 4 As in the first procedure a second extraction of the gland residue was made with 0 1 N HCl at room temperature, and two fractions were derived from this extract adjusted to pH 3 at 12 per cent and at 96 per cent saturation with sodium chloride In this procedure nearly all of the growth promoting activity of the primary extract is concentrated in a precipitate obtained at 2 M ammonium sulphate at pH 7 This fraction (AS pH 7) derived from 45 g of fresh glands weighed 295 mg Its growth activity was estimated to be 0 55 units/mg and it represents a yield of only 3 6 g /kg much below the expected yield of growth hormone from these glands The growth activity of the acid extract was not measured in this experiment since we were not at the time aware of the possibility that most of the growth hormone was left in the gland residue and could be extracted with dilute acid

Satisfactory recovery of the activity from the 2 M ammonium sulphate fraction was achieved by fractionation with ethanol from dilute KCl solution at pH 7 2 (Table IV)

Neither of these procedures appeared to be satisfactory from the point of view of obtaining good yields of growth hormone and at the same time allowing the isolation or concentration of other activities

by dialysing and lyophilizing the whole supernatant solution It will be seen from Table III that a very large residue is left

Table III

RECOVERY OF GROWTH HORMONE FROM FRACTION F

Fraction F (1 92 g , 806 units)

Fraction	*Weight (mg)*	*Units/mg*	*Total units*
R	1 380	—	—
A	264	1 2	297
B	—	—	—
Fraction R (1 38 g)			
C	1 114	nil	—
D	266	1 7	452
Total			749

R insoluble in 0 1 M ammonium sulphate pH 7 A precipitate at 2 M ammonium sulphate pH 7 B soluble in 2 M ammonium sulphate pH 7 C insoluble in 0 1 M KCl pH 6 8 D precipitate at 2 M ammonium sulphate pH 6 8

and that, although the 2 M ammonium sulphate fraction, A, is highly active, it represents only a small part of the estimated total activity of the fraction It was discovered that the insoluble fraction R would dissolve completely in 0 1 M KCl at pH 11 From this solution a heavily coloured inert fraction precipitates at pH 6 8 A highly active growth promoting fraction is obtained from the clear supernatant solution at 2 M ammonium sulphate and pH 6 8 Of the total of 806 units of activity estimated to be present in the original fraction F the two fractions account for 749 or 92 per cent, a very satisfactory accounting It will be noted that both products appear to have been more active than the standard, so that the weight yield of the fractions is considerably less than the yield in units of activity This phenomenon has been noted several times in the course of this work

One may speculate that, if each molecule of growth hormone is a single active unit, then when equal weights of purified human and bovine growth hormone are compared the potencies should be in the inverse ratio of the molecular

During the past 18 months my colleague Dr Stanley Ellis has been working on a further development of methods of fractional extraction of ox sheep and pig pituitaries He has evolved a new procedure (Ellis 1960) by which several of the major active principles of the anterior lobe can be largely separated from one another Three successive extractions are carried out

A water, at pH 5 5
B 0 1 M ammonium sulphate at pH 4 0
C 0 25 M ammonium sulphate at pH 7 5

As an illustration of the results of this procedure a summary of the distribution of the activities in the three extracts of sheep pituitaries is presented in Fig 1 It will be seen that FSH is the principal activity found in extract 'A TSH and LH the main activities in extract 'B ' and growth hormone in extract C ACTH and prolactin appear to be absent from these three extracts The yields of activities in the three extracts are satisfactorily high The results with ox and pig pituitaries were qualitatively similar These initial separations greatly facilitate the subsequent purification of the active principles and they reduce the chances of cross contamination Both FSH and growth hormone (GH) have been carried to high degrees of purification with excellent recoveries from their respective primary extracts LH and TSH are quite easily concentrated from extract B but their clean separation has not been achieved

This new procedure has been applied to human pituitaries with interesting but decidedly different results Tables V–VII summarize the observations that have so far been made on extracts A ' B and ' C in two experiments with acetone dried material and one experiment with fresh frozen glands From the data of the three Tables it may be seen that FSH appears to be concentrated in the 'A' extract and that the specific activity and the total indicated quantity are high The greater part of the LH also appears in the "A extract TSH is about evenly divided between the A ' and 'B

Table IV

RECOVERY OF GROWTH HORMONE FROM FRACTION AS pH 7

Fraction AS pH 7 (295 mg 161 units)

Fraction	*Weight (mg)*	*Units/mg*	*Total units*
A	128	—	—
B	106	1 0	106
C	61	—	—

A insoluble in 0 1 M KCl pH 4 5 B precipitate from 25 per cent ethanol pH 7 4 C precipitate from 55 per cent ethanol pH 5 5

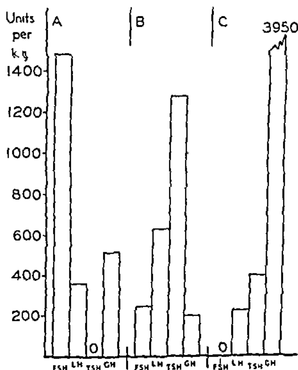


FIG 1 Distribution of FSH LH TSH and GH activities in three successive extracts of fresh whole sheep pituitaries Extract A water pH 5 5 B 0 1 M ammonium sulphate pH 4 0 C 0 25 M ammonium sulphate pH 7 5 No ACTH or prolactin were found in these three extracts (Ellis 1960)

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extracts, but is more highly concentrated in the smaller extract "B"

It was a surprise to find significant amounts of growth hormone in the first water extract In the case of the fresh

Table V

HORMONAL ACTIVITIES DERIVED FROM HUMAN PITUITARIES BY EXTRACTION WITH WATER AT pH 5 5 (EXTRACT "A")

	a_1	a_2	f
Solids g /kg	13 6	5 7	17 4
FSH units/mg	1 0	1 9	0 75
units/kg	13 700	10 900	13 000
LH units/mg	—	—	0 053
units/kg	—	—	920
TSH units/mg	0 02	—	0 013
units/kg	342	—	2[illegible]6
GH units/mg	0 38	0 20	(1 73)
units/kg	5 170	1 1[illegible]0	(30 000)
Prolactin units/mg	0 8	—	—
units/kg	11 300	—	—
ACTH units/mg	—	0 37	—
units/kg	—	2 116	—

a_1 a_2 acetone dried glands f fresh frozen glands
The weight reference is to fresh gland weight

Table VI

HORMONAL ACTIVITIES DERIVED FROM A SECOND EXTRACTION OF HUMAN PITUITARIES WITH 0 1 M AMMONIUM SULPHATE pH 4 0 (EXTRACT "B")

	a_1	a_2	f
Solids g /kg	4 24	5 15	8 78
FSH units/mg	0 27	0 40	0 20
units/kg	1 150	2 060	1 756
LH units/mg	—	—	0 064
units/kg	—	—	850
TSH units/mg	0 10[illegible]	—	0 050
units/kg	444		439
GH units/mg	0 30	0 22	0 36
units/kg	1 270	1 140	3 100
Prolactin units/mg	0 19	—	—
units/kg	800	—	—
ACTH units/mg	0 14	—	—
units/kg	589	—	—

(Symbols as in Table V)

Table VII

HORMONAL ACTIVITIES DERIVED FROM A THIRD EXTRACTION OF HUMAN PITUITARIES WITH 0 25 M AMMONIUM SULPHATE pH 7 5 (EXTRACT C)

	a_1	a_2	f
Solids g /kg	8 17	8 41	13 16
FSH units/mg	0 07	—	0 02
units/kg	577	—	263
LH units/mg	—	—	0
units/kg	—	—	0
TSH units/mg	0 025	—	0 005
units/kg	206	—	66
GH units/mg	0 46	0 81	0 53
units/kg	3 730	6 "80	6 900
Prolactin units/mg	4 1	—	—
units/kg	33 400	—	—
ACTH units/mg	0 02	—	—
units/kg	188	—	—

(Symbols as in Table V)

glands the specific activity of the A extract (units/mg) was already greater than that of the standard and the cal culated total yield was fantastically high It is possible that this extract contains an optimal mixture of activities for the promotion of growth in the hypophysectomized rat if so one might have expected to see a similar effect in the two experiments with acetone dried material It is also possible that the yield of hormone from fresh material can in fact be much greater than that from acetone dried glands This point will be examined when more fresh glands are available Finally the possibility of error in the assay must be admitted A careful examination of all of the data of the assay and of the history of the animals revealed no obvious error There was unfortunately no opportunity to repeat this assay A relatively small amount of growth promoting activity was found in the 'B" extract and a good concentration was found as expected in the C extract in all the experiments

The distribution of prolactin appears roughly to follow that of growth hormone A significant amount appears in extract A very little in extract B and about two thirds of the total in extract C

Finally, it is noted that a significant amount of ACTH appears in extract "A". The "B" and "C" extracts contain additional small amounts of this activity at low concentration.

In each of these experiments, a fourth ("D") extract was prepared. For experiments a_1 and f the gland residue was extracted in 0.1 N HCl at room temperature for 24 hours and the extract was fractionated with sodium chloride at pH 3 as already described. In the experiment a_2 the residue was extracted with alkaline ethanol according to the method of Bates and Riddle (1935) for prolactin, a single fraction was

Table VIII

HORMONAL ACTIVITIES DERIVED FROM A FOURTH EXTRACTION OF HUMAN PITUITARIES WITH (I) N HCl OR (II) ALKALINE 60 PER CENT ETHANOL (EXTRACT "D")

		a_1	a_2	f
Fraction DA				
Growth hormone	units/mg	0.54	—	0.10
	units/kg	1 840	—	630
Prolactin	units/mg	0.8	1.5	0
	units/kg	2 200	5 500	0
Fraction DB				
ACTH	units/mg	1.0	—	1.0
	units/kg	3 200	—	2 [illegible]00

(Symbols as in Table V.)

For a_1 and f fraction DA is precipitated at pH 3, 12 per cent saturation with NaCl; fraction DB the precipitate at 96 per cent saturation with NaCl. For a_2 fraction DA is precipitated from 83 per cent ethanol, pH 5.5

obtained from the extract at pH 5.5 and 83 per cent ethanol. A summary of the activities found in these "D" extracts is presented in Table VIII. A small amount of growth promoting activity is present in the acid extracts. Similarly, prolactin was present both in the acid extract of one experiment (a_1) and in the alkaline ethanol extract of experiment a_2. The final acid extract of the fresh glands appeared to contain little growth activity and no prolactin. The fraction obtained from the acid extract at pH 3 and 96 per cent saturation with sodium chloride contained ACTH at a concentration of 1 unit

per mg More than half of the total ACTH is present in this fraction

This method of fractional extraction which Ellis (1960) has found works so well with fresh whole pituitaries from sheep ox and pig does not yield the same distribution of activities with either fresh or acetone dried human pituitaries Both ACTH and prolactin appear in significant amounts in one or another of the first three extracts LH appears mainly in extract A' rather than in B and it is not concentrated favourably in either extract Growth hormone appears in high proportion in the first water extract at pH 5 5 instead of being concentrated in the C extract TSH and FSH are the only two activities that appear to be distributed as Ellis found them in the glands of other species The indicated FSH activity is high much higher than that found in the glands of other species of animals It is however of the same order as that reported by Steelman Segaloff and Mays (1958) in their preparation of gonadotropins from human pituitaries

This procedure falls far short of the ideal of a convenient separation of every major activity in good yield We are now trying to modify the method mainly to concentrate the growth hormone in one fraction Our first attempt involved extracting an acetone powder of the glands first with 0 25 M ammonium sulphate at pH 4 and then with 0 25 M ammonium sulphate at pH 7 5 The growth hormone activity was distributed as before in both extracts It was interesting to note however that both TSH and LH were found in the first extract in higher concentration and greater total amount than in any previous experiment The data are summarized in Table IX Experimental work on this problem is still being carried on

Although the observations on each extract are not as complete as one would like the fairly exhaustive extraction of several small lots of glands allows us to make a rough estimate of the total amounts of each activity that may be found in the human pituitary These estimates are presented in Table X

Table IX

MODIFIED FRACTIONAL EXTRACTION OF HUMAN PITUITARIES

	GH		*LH*		*TSH*	
	units/mg	*units/kg*	*units/mg*	*units/kg*	*units/mg*	*units/kg*
Extract A	0 63	10 600	0 2	3 360	0 2	3 3[illegible]0
Extract B	0 47	4 980	0 0[illegible]	212	0 0[illegible]	[illegible]12

A 0 2[illegible] M ammonium sulphate pH 4 0 B 0 2[illegible] M ammonium sulphate pH 7 [illegible]

Table X

ESTIMATED TOTAL ACTIVITIES EXTRACTED FROM HUMAN PITUITARIES

	Units per kg fresh weight
Growth hormone	12 000 (17 800)*
Prolactin	49 000
FSH	14 000
LH	2 [illegible]00
TSH	1 700
ACTH	6 000

* Figure in parentheses includes high estimate from fresh pituitaries (Table V)

It is gratifying to find a good even in some instances a higher than ordinary amount, of all the activities in human pituitaries collected at autopsy and preserved under not always favourable conditions This makes worth while the attempt to separate all of the activities in good yield so that the studies which have so far been made possible on human growth hormone and FSH can be extended to the other active principles of the human pituitary

Summary

Attempts have been made to devise methods for the fractionation of human pituitaries which will permit the economical separation and the isolation or concentration of all of the active principles of the anterior lobe It is shown that the method of Wilhelmi Fishman and Russell (1948) fails of this purpose Trials of a method of fractional extrac

tion applied successfully by Ellis (1960) to bovine ovine and porcine pituitaries reveal that the procedure does not succeed as well with human pituitaries It does however give promise that an efficient general method of fractionation of human pituitaries can be evolved since the yields of the six major hormones of the anterior lobe are satisfactorily high

Acknowledgments

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The prolactin standard used was the preparation NIH SP 1 distributed by the Endocrinology Study Section National Institutes of Health

REFERENCES

BATES R W and RIDDLE O (1935) *J pharmacol exp Ther* 55 365

BECK J C MCGARRY E E DYRENFURTH I and VENNING E H (1957) *Science* 125 884

BONSNES R W and WHITE A (1940) *Endocrinology* 26 990

ELLIS S (1958) *J biol Chem* 233 63

ELLIS S (1960) In preparation

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FEVOLD H L LEE M HISAW F L and COHN E J (1940) *Endocrinology* 26 999

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LAMBERG B A (1953) *Acta med scand* suppl 279

LEWIS U J and BRINK N G (1958) *J Amer chem Soc* 80 4429

LI C H (1952) *Acta endocr (Kbh)* 10 255

LI C H (1955) *J biol Chem* 211 555

LI C H EVANS H M and SIMPSON M E (1945) *J biol Chem* 159 353

LI C H and PAPKOFF H (1956) *Science* 124 1293

MARX W SIMPSON M E and EVANS H M (1942) *Endocrinology* 30 1

RABEN M S (1957) *Science* 125 883

RABEN M S (1959) *Recent Progr Hormone Res* 15 71

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Extract A	0 63	10 600	0 2	3 360	0 2	3 360
Extract B	0 47	4 980	0 02	212	0 02	212

A 0 25 M ammonium sulphate pH 4 0 B 0 25 M ammonium sulphate pH 7 5

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RUSSELL J A and WILHELMI A E (1958) *Ann Rev Physiol* 20 43
SAYERS M A SAYERS G and WOODBURY L A (1948) *Endocrinology* 42 379
SHORR E, CARTER A C SMITH R W Jr KENNEDY B J HAVEL R J ROBERTS T N SONKIN L L and LIVINGSTONE E T (1955) *In* Hypophyseal Growth Hormone Nature and Actions p 522 eds Smith R W Jr, Gaebler O H and Long C N H New York McGraw Hill
STEELMAN S L and POHLEY F M (1953) *Endocrinology* 53 604
STEELMAN S L SEGALOFF A and MAYS M G (1958) *Arch Biochem* 78 262
WILHELMI A E (1955) *In* Hypophyseal Growth Hormone Nature and Actions p 59 eds Smith R W Jr Gaebler O H and Long C N H New York McGraw Hill
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DISCUSSION

Loraine Is there any significant deviation from parallelism in any of these assays or are the slope differences insignificant?

Wilhelmi The parallelism is very good except in one instance i e when there is an overwhelming amount of FSH present in the preparation then the slope falls and the test curve is no longer parallel to the unknown But there has to be a very large preponderance and the method is so sensitive that one can use quite small doses and usually avoid this effect

Loraine Is the method you mention more sensitive than the ventral prostatic weight test in hypophysectomized rats?

Wilhelmi Yes, Dr Ellis obtains measurable response with less than 1 µg of standard—the standard is the Armour LH 227-80 preparation

Loraine Are you satisfied that the technique described by Steelman and Pohley (1953 *loc cit*) is indeed specific for FSH activity? Have you compared results by this method with those obtained by the technique described by Evans and his co workers and depending on follicular development in the ovaries of hypophysectomized immature female rats (Evans H M Simpson M E Tolksdorf S and Jensen H (1939) *Endocrinology* 25 529)?

Wilhelmi In any method of measuring gonadotropin there are distinct limits to one s satisfaction It is quite possible that where, for example as in the case of the human pituitary and in crude fractions of the extracts there is also present a significant amount of LH which is added to the chorionic gonadotropin base this changes the character of the response so that some of our estimates of FSH activity in these crude systems are undoubtedly high However if

we go on fractionating these extracts we can then separate these activities largely and account for a significant proportion of the initially observed FSH activity in the derived fraction This makes me a little more confident that we have not got a gross exaggeration of response in the assay Secondly with reference to comparing it with other methods we have not done that

Raben Prof Wilhelmi your estimate of the growth hormone content of the human pituitary seems to be a minimal one because one can easily obtain 5 per cent of the dry weight as growth hormone and there is some loss in the process I would have guessed that there is more there

Wilhelmi This was in terms of 12 g /kg of fresh weight which works out roughly at 60 g /kg of acetone dried weight that is about 6 per cent of the dry weight This is the same order as you have recorded before

As to the potency of the fractions these activities too are in bovine units This is my speculation we have two hormones in the molecular ratio of 25 000 47 000 if one were to argue that there is one active unit per molecule then the relative potencies of two perfectly pure preparations all other factors being equal would be in inverse ratio to the molecular weights so that a molecule of purified human growth hormone should be 1 7 times as active as the bovine standard This means that the estimated quantities of hormone in the gland are nearly twice the actual quantity in the gland which comes closer to reason I do not know if that is a legitimate speculation about the unit activity per molecule and about the expected molecular weight ratios but in our most purified preparations we approached these ratios in the growth assay Prof Li in his reports on activity has never mentioned this Have you observed that Prof Li?

Li Your figure Prof Wilhelmi is a little higher than my estimate Dr Gemzell and I published an estimate of the content of growth hormone in a single human pituitary arrived at by physicochemical means as well as by bioassay (Gemzell C A and Li C H (1957) *J clin Endocr* 28 149) Our estimate was on the average 5 mg /g In recent experiments in which immunological methods were used this previous value was confirmed (Li C H Moudgal N R and Papkoff H (1960) *J biol Chem* in press) Again an average of approximately 5 mg /g was reported Thus I feel that the estimates of 12 mg /g reported by Prof Wilhelmi and of 20 mg /g reported by Dr Raben are somewhat too high

Wilhelmi The immunological method will probably estimate human growth hormone in its own terms and then if you cut my figure in half we are in agreement but in bovine units this is a little

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differences were present (Gaddum J H (1955) *In* Polypeptides which Stimulate Plain Muscle p 153 Edinburgh Livingstone)

Wilhelmi I think that is something we must do

Li We have compared the results of the tibia test with those of the immunological test (although not with those of the weight gain test) The two methods were in good agreement

Prof Wilhelmi what is your estimate of the activity of the lactogenic hormone in the human pituitary ?

Wilhelmi 49 000 units/kg fresh weight

Li Would you assume that this would represent about 25 units/mg since it is highly purified material ? Then you have about 2 g

Wilhelmi It is 2 g /kg of fresh weight It agrees remarkably well with an estimate that Prof Young with Chance and Rowlands made a very long time ago (Chance M R A Rowlands I W, and Young F G (1939) *J Endocr* 1 239)

Li Does that mean that Prof Young and Prof Wilhelmi agree that the active purified fraction of prolactin in the human has a specific activity of perhaps about 25 units/mg ?

Wilhelmi On the basis of our present assay it would be something like 20–30 units/mg

nearer to what we see Secondly a very interesting question arises the tibia test if one is to believe the observation that effects of growth hormone directly say on sulphur uptake in the cartilage are not obtained with growth hormone *in vitro*, may be an indirect test of growth hormone activity One wonders if it gives a maximal effect as compared to the weight gain test Have you ever seen what one might call the expected ratio of potencies of pure human as opposed to pure bovine growth hormone? It is about 1 71 1 reckoned by other arguments

Li The assay method based on the body growth in hypophysectomized animals is not I feel very useful for human growth hormone We have found that when one injects human growth hormone into 28 day old hypophysectomized rats once daily for 10 days the weight gain elicited by the growth hormone will begin to decline toward the end of that interval and indeed after 10 or 15 days of injection no matter how large a dose is given the animals become entirely resistant to both human and monkey growth hormone (Li C H Papkoff H and Jordan C W (1959) *Proc Soc exp Biol (N Y) 100 44)* *For these primate hormones* we feel that the body growth measured by the weight gain in hypophysectomized animals within 10 days may furnish an estimate of only the lowest limit of the growth hormone effect and after 10 days it is of absolutely no use because of this plateauing of effect

Wilhelmi I do not agree with you about the development of resistance We are using Sprague Dawley rats and in these the resistance does not appear to develop so rapidly In every instance we make sure that in the assay the slope and the parallelism are satisfactory Occasionally we see in some rats that with monkey and human growth hormone the test substance gives a slope markedly different from the standard but in most instances they agree very well However you cannot use hypophysectomized rats that have been *given monkey or human growth hormone* in a second assay the response will be nil But you can still get effective response with bovine or pig growth hormone The question of resistance is a real one but I still prefer the weight gain test in the animal where it is possible to do it and exercise appropriate control of the slope of the response

Loraine I wonder if either Prof Li or Prof Wilhelmi has performed an experiment in which the same extract was assayed for growth hormone activity by different methods For example have any comparisons been made between the tibial test and the immunological method or between the immunological method and the body weight method? In this way it would be possible to calculate the index of discrimination and to determine whether or not qualitative

Table I

SOME CHARACTERISTICS OF VARIOUS PITUITARY GROWTH HORMONES

Properties	*Beef*	*Sheep*	*Pig*	*Whale*	*Monkey*	*Human*
Molecular weight	45 000	48 000	41 000	40 000	5 000	[illegible] 000
Isoelectric point, pH	6 8	6 8	6 3	6 [illegible]	5 5	4 9
-S-S- bridges	4	5	3	3	4	
N Terminal sequence	Phe Thr Ala Ala Phe Al	Phe Ala	Phe	Phe	Phe	P[illegible]e Ser Thr
C Terminal sequence	Leu Ala Phe Phe	Ala Leu Phe	(Leu,Ala Phe) Phe	Leu Ala Phe	(Ala Gly) Phe	Leu Phe

STUDIES ON HUMAN PITUITARY GROWTH AND GONADOTROPIC HORMONES

Choh Hao Li

Hormone Research Laboratory University of California Berkeley

It was demonstrated as early as twenty years ago that bovine and ovine lactogenic hormone preparations differ with respect to their tyrosine content (Li, Lyons and Evans, 1940) and their solubility behaviour (Li, Lyons and Evans, 1941) That a protein hormone prepared from one species may not be identical with the same hormone isolated from another species was later confirmed by investigations on ovine and porcine gonadotropins (Chow 1942, Van Dyke, Pan and Shedlovsky, 1950) It was therefore not surprising when in 1956 human growth hormone was found to possess physicochemical characteristics that are markedly different from the growth hormone isolated from beef glands (Li and Papkoff 1956) There are now growth hormone preparations from six different species namely beef sheep whale, pig, monkey and man that can be obtained in a high degree of purity for physicochemical studies Table I summarizes some of the relevant data * which indicate that even the hormone preparations obtained from species as closely related as man and monkey are not identical The present report deals only with investigations on human pituitary growth and gonadotropic hormones currently being carried out in this laboratory, and is not intended to be a thorough review of the field nor to cover the literature

* The data for the porcine growth hormone have not been published before (Papkoff and Li unpublished work however it was reported in July 1959 at the Gordon Conference on Proteins and Nucleic Acids)

Table III

ASSAY OF GROWTH HORMONE FROM HUMAN PITUITARIES IN HYPOPHYSECTOMIZED FEMALE RATS*
FOR BIOLOGICALLY ACTIVE CONTAMINANTS

Group	Body weight			Adrenal		Thyroid		Ovaries	
	Onset	Autopsy	Gain	Weight	Histology	Weight	Histology	Weight	Histology
	g	g	g	mg		mg		mg	
Experimental 0 50 mg growth hormone total dose	70	75	5	12 2 ± 0 9†	atrophic	5 3 ± 0 7	atrophic	9 1 ± 1 7	small follicles slight interstitial cell stimulation
Control saline	69	69	0	11 5 ± 1 0	atrophic	4 7 ± 0 6	atrophic	8 4 ± 1 9	small follicles interstitial tissue deficiency

* Female rats hypophysectomized at 3 days of age and injected 7 days later subcutaneously once daily for a period of 3 days 4 animals group
† Mean ± s e

Growth-promoting activity of human growth hormone on experimental animals

Before the growth promoting activity of human growth hormone (HGH) in hypophysectomized rats and mice is discussed, it is important to establish that the preparation is free from contamination with other active components By standard bioassay methods for the detection of thyrotropin (TSH) and adrenocorticotropin (ACTH), the preparation was found to contain less than 0 3 U S P units of ACTH and approximately 0 02 U S P units of TSH per mg of the hormone protein (Table II) That the preparation is almost

Table II

BIOASSAY OF HUMAN GROWTH HORMONE FOR THYROTROPIC AND ADRENOCORTICOTROPIC CONTAMINATION

Preparations	*Contaminating activities U S P unit/mg*	
	*Thyrotropin**	*Adrenocorticotropin†*
L 2783B	0 013	< 0 3
I 2798B	0 016	
L 2744G	0 035	

* We are indebted to Dr R W Bates of the National Institutes of Health for these values the assay was carried out by the method of Bates and Cornfield (1955)
† We are indebted to Dr S W Hlers of the Wilson Laboratories for the assay whi h was carried out by the U S P intravenous ACTH assay method

entirely free from these two hormones as well as from any gonadotropins was confirmed by histological examination of the endocrine organs in hypophysectomized rats which had been injected with a total dose of 0 5 mg of HGH over a period of 8 days (Table III)

The results of bioassay of the growth promoting activity of HGH in hypophysectomized rats by the tibia test (Greenspan *et al* 1949) can be seen in Table IV A straight line relationship was evident from a plot of the dose response curve according to the equation

$$W = 42\ 5 \log D + 184\ 4$$

to the tibia is calculated in accordance with this equation to be 135 What the significance of this number may be remains to be investigated, the salient fact here is that the adherence to linearity on the part of the assay data obtained for HGH by the tibia test when the relationship of dose to response is plotted according to Stetten s equation implies that binding of the hormone on to the active site or sites on the target organ in this instance the tibial cartilage by some mechanism

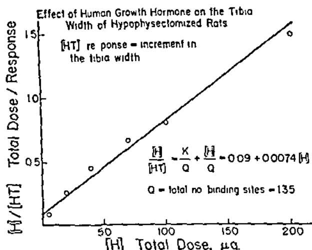


FIG 1 Dose response curve of HGH according to the tibia test in hypophysectomized rats (for the data see Table IV)

(such as a key–keyhole or enzyme–substrate attachment) is necessary for the biological activity to be exercised

Human growth hormone has also been shown to be active in hypophysectomized mice according to the tibia test (Table V) (Lostroh and Li 1957*b*) when the assay data are compared with those obtained for the bovine growth hormone (Lostroh and Li 1957*a*) no differences in capacity to stimulate tibial width are observed between the hormones from the two species

Table IV

BIOASSAY BY THE TIBIA TEST OF GROWTH HORMONE ISOLATED FROM HUMAN PITUITARY GLANDS

Total dose	No. of rats	Width of tibial epiphysial cartilage plate	
		Mean ± S.E.	Difference from control
µg		micra	micra
0	10	157 ± 2	0
5	18	213 ± 3	56
20	16	234 ± 4	77
40	24	246 ± 3	89
70	7	261 ± 3	104
100	7	280 ± 1	123
200	8	291 ± 2	134

where W is the width of tibial epiphysial cartilage in micra and D the total dose in micrograms. It is of interest that when the total dose was plotted against the ratio of total dose to response, a straight line relationship was also observed (Fig. 1). An equation expressing this relationship of dose to dose response in connexion with the biological effect of a hormone was recently proposed by Stetten (1950) as

$$\frac{[H]}{[HT]} = \frac{K}{Q} + \frac{[H]}{Q}$$

where $[H]$ represents the level of circulating hormone, a quantity which is directly proportional to the total dose of the hormone administered; $[HT]$ is the number of sites at which a hormone attaches itself on to a target organ, a quantity which is directly proportional to the response;* Q is the total number of binding sites available for such attachment, and K is the equilibrium constant given by the expression $K = [H][T]/[HT]$, in which $[T]$ is the total number of available sites not occupied. From the slope of the curve in Fig. 1 the total number of sites at which the hormone is bound on

* For the tibia test, the response is defined as increment in the tibial width over the control.

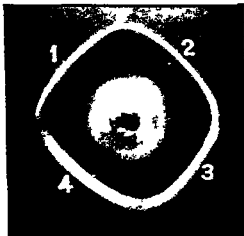


FIG 2. Interaction of antiserum to HGH with varying concentration of human growth hormone as determined by the Ouchterlony test

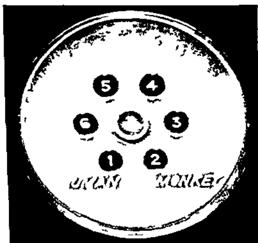


FIG ? Interaction of antiserum to HGH with purified preparation from various species as determined by the Ouchterlony test. Central well contains antiserum to HGH; well 1-6 contain 10 μg each of human, monkey, porcine, whale, ovine and bovine growth hormone respectively

f n a p n 5

Table V

THE TIBIAL RESPONSE OF THE HYPOPHYSECTOMIZED FEMALE C3H MOUSE TO GROWTH HORMONE FROM HUMAN PITUITARIES

Daily dose	*No of animals**	*Body weights*			*Width of tibial epiphysial cartilage plate*
		Day of operation	*Day of first injection*	*At autopsy*	
µg		g	g	g	micra
0	10	18	12 5	14 5	58 0 ± 1 1†
1	8	16	13	14	87 0 ± 3 0
5	7	17	13	16	119 8 ± 3 5
10	7	16	13	15 5	128 6 ± 3 1

All animals hypophysectomized at 35 days of age and sacrificed at 6 days Injections begun at 47 days of age and continued for 17 days
† Mean ± S E

The growth promoting activity of HGH has also been determined by the criterion of increment in body weight of hypophysectomized rats In a 10 day test a daily dose of 0 005 mg of HGH produces an increment of 12 2 g in body weight over that of the controls (Table VI) however, if the

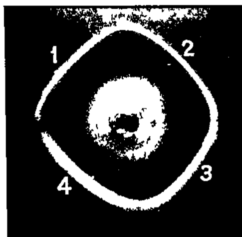
Table VI

BIOASSAY OF HUMAN GROWTH HORMONE ACCORDING TO BODY WEIGHT INCREMENT PRODUCED IN HYPOPHYSECTOMIZED RATS

Daily dose	*No of rats**	*Body weight (mean ± S.E)*		
		Onset	*Final*	*Gain in 10 days*
µg		g	g	
0	8	70 4 ± 1 8	72 1 ± 2 1	1 6
5	7	72 1 ± 2 6	85 9 ± 3 2	13 8
20	7	69 9 ± 1 8	89 0 ± 2 0	19 1

Hypophysectomized female rats (8 days old at operation) were injected intraperitoneally with 0 5 ml of solution once daily for 10 days beginning on the fourteenth postoperative day

injections were continued for a longer interval the animals became resistant to the hormonal effect This is also true of monkey growth hormone In contrast whale and bovine growth hormones were found to be capable of promoting a



I ic – Inter iction of anti erum to HGH with varyin concentration of human growth hormone as determined by the Ouchterlony test

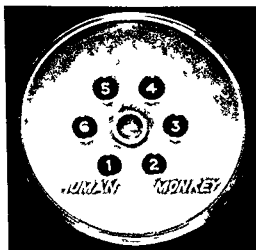


Fig 3 Interaction of anti erum to HGH with purifed preparations from various pecies as determined by the Ouchterlony test Central well contains antiserum to HGH wells 1–6 contain 10 µg each of human monkey porcine whale ovine and bovine growth ho mone respectively

f n g paj 5

continuous gain in body weight for as long as 90 days (Li, Papkoff and Jordan, 1959) This difference in biological behaviour between the primate growth hormones on the one hand and the bovine or whale growth hormones on the other may be due to their differences in antigenicity, for it has been shown that human growth hormone is a far better antigen than is the hormone from beef glands (Hayashida and Li 1959)

Immunological behaviour of HGH (With R Moudgal, H Papkoff and T Hayashida)

Earlier immunological studies conducted with bovine (Hayashida and Li 1958*a*) and human (Hayashida and Li 1958*b*) growth hormones demonstrated that the protein hormone forms antibodies specific to the species as evidenced by results of precipitin ring tests with rabbit antiserum and anaphylactic shock experiments in guinea pigs It has been further demonstrated in hypophysectomized rats that the rabbit antiserum possesses specific anti hormone activity In addition the immunological homogeneity of the rabbit antiserum to human growth hormone has now been indicated by agar gel diffusion tests performed by the Ouchterlony technique (Fig 2) It is interesting that not only the human growth hormone but also the monkey growth hormone gave a single continuous reaction line with the antiserum to human growth hormone indicating a 'reaction of identity' on the part of the primate hormones (Fig 3)

We have recently obtained a purified gamma globulin pre paration from rabbit antiserum to human growth hormone by means of a modification of the rivanol procedure (Horejsi and Smetana 1956) and as expected antibodies to human growth hormone were concentrated in the gamma globulin With the gamma globulin used as the antibody quantitative precipitin tests *were performed on human and monkey* growth hormones It was noted that the hormones from both species had equivalence points at 70 µg when 6 mg of gamma globulin containing antibody were employed but the ratios

of antibody to antigen were different for each of these two proteins (Fig 4) This difference is not surprising in view of their differing physicochemical properties, as shown in Table I

In addition, we have estimated the growth hormone content in a single human pituitary on the basis of this quantitative

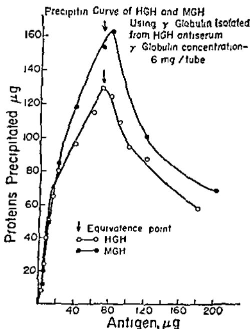


FIG 4 Quantitative precipitin tests of HGH and monkey growth hormone (MGH) with gamma globulin prepared from rabbit anti serum to HGH

precipitin curve obtained from the reaction of the gamma globulin with the purified human growth hormone In a typical experiment one pituitary (648 mg) was homogenized in a Waring Blendor with 50 ml of cold distilled water for 2 minutes The mixture was transferred to a beaker the pH was adjusted to 10 5 and the solution was stirred at 0° for 1 hour After centrifugation the clear supernatant fluid

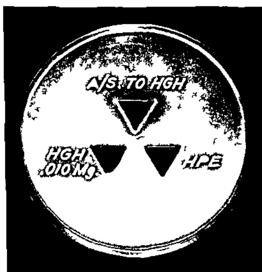


Fig 5 Interaction of antiserum (A/S) to HGH with crude human pituitary extract (HPE) as determined by the Ouchterlony test

of antibody to antigen were different for each of these two proteins (Fig 4) This difference is not surprising in view of their differing physicochemical properties, as shown in Table I

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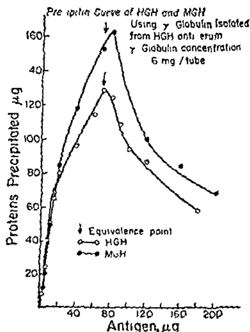


FIG 4 Quantitative precipitin tests of HGH and monkey growth hormone (MGH) with gamma globulin prepared from rabbit antiserum to HGH

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was diluted to make a final volume of 100 ml, and the pH was adjusted to 7 0 Varying amounts of the extract were allowed to react with the gamma globulin the precipitates were analysed and the results were compared with the above purified human growth hormone precipitin curve It was estimated that the extract from a single human pituitary contained 5 mg of growth hormone an estimate which is in good agreement with the value previously reported (Gemzell and Li 1958)

Finally, we have employed the immunological method to elucidate the question of identity between the isolated human growth hormone and the hormone as it exists in its crude state An agar gel diffusion test was carried out according to the Ouchterlony procedure, with triangular wells One well contained 0 3 ml of antiserum to rabbit growth hormone the other two wells contained the antigens namely 0 2 ml of human growth hormone (10 μg) and 0 2 ml of the crude pituitary extract (Hayashida and Li 1959) A single reaction line appeared between the well containing the antiserum and each of the wells containing the two antigens (Fig 5) suggesting a homogeneous antigen antibody system in both instances Moreover the complete fusion of these two lines suggests that there is in the crude human pituitary extract a substance which is immunologically indistinguishable from the isolated human growth hormone

Purification of human pituitary follicle stimulating hormone (With Ute Groschel)

From a CaO extract of acetone dried lyophilized or frozen human pituitary glands * a procedure has been developed for the purification of growth hormone prolactin † follicle stimulating hormone (FSH) and interstitial cell stimulating

* For a high yield of growth hormone frozen glands are preferable Acetone dried pituitaries give the lowest yield of both growth and gonadotropic hormones

† The purification and properties of prolactin from human pituitary glands will be reported elsewhere

Table VII

YIELD AND POTENCY OF FRACTIONS OBTAINED FROM PURIFICATION PROCEDURE OF HUMAN FSH

Fraction	*Weight*	*Procedure*	*Activity*
	mg		units/mg
A	10 000	Lyophilized whole gland	
SII	2_0	$(NH_4)_2SO_4$ fractionation	0
G	60	Column electrophoresis	30
H	2_	DEAE cellulose chromatography	100

biological activity can be achieved with the FSH fraction SII used as starting material An average yield of 25 mg of highly purified FSH (fraction H) can be obtained from 10 g of lyophilized human pituitaries this preparation appeared

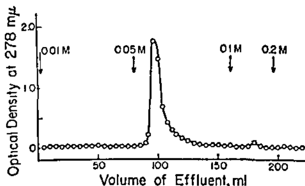


FIG 7 Chromatography of human FSH fraction H (4? mg) on DEAE cellulose equilibrated with 0 01 M K_2HPO_4

to be chromatographically homogeneous on a DEAE cellulose column (Fig 7)

FSH (fraction H) has been assayed in graded doses by the augmentation test the data may be expressed by the equation

$$A = 29\ 6 + 3\ 1\ D$$

hormone (ICSH) concentrates (Fig 6) The isolation of growth hormone (Li and Papkoff 1956, Li, 1957) and the partial purification of FSH (Li 1958) have already been described

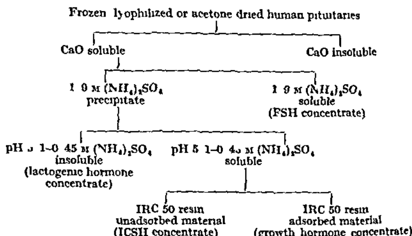


FIG 6 A scheme for the purification of human pituitary hormone concentrates

The further purification of FSH can be achieved by submitting FSH fraction SII to column electrophoresis in a 0 1 M $NaHCO_3$ Na_2CO_3 buffer of pH 8 6 with ethanolized cellulose as the inert support (Flodin and Kupke 1956 Porath 1956) The active fraction obtained by this column electrophoresis was then applied on to a column containing diethylaminoethyl (DEAE) cellulose (equilibrated with 0 01 M K_2HPO_4) and stepwise elution was performed with 0 05 M 0 10 M and 0 20 M solutions of the phosphate buffer The FSH activity* was located in the 0 05 M eluate Table VII summarizes the steps in the purification of the FSH and gives the yield and potency of each fraction a tenfold increase in

* The assay method based on the augmentation of ovarian weight effected by FSH in combination with human chorionic gonadotropin (HCG) in intact rats was used for estimating the follicle stimulating potency (Steelman and Pohley 1953 Li 1958) A unit of FSH activity is defined as that dose which elicits an increase in ovarian weight of 100 per cent over the HCG control

then submitted to electrophoretic fractionation on a cellulose column the peak with the lower mobility (designated fraction HB) contained the bulk of the ICSH activity Further purification of fraction HB was achieved by chromatography on an Amberlite IRC 50 resin column For this resin column chromatography 119 mg of fraction HB were submitted to chromatography on a column containing 100 ml of IRC 50 resin equilibrated with 0 2 M potassium phosphate buffer of

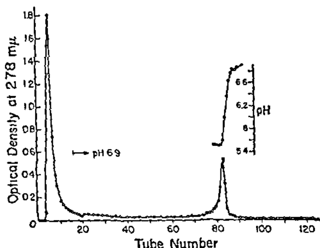


FIG 8 Chromatography of human ICSH fraction HB (110 mg) on IRC–50 re in equilibrated with 0 2 M phosphate buffer of pH 5 51 Elution with 0 2 M phosphate buffer of pH 6 90 begun at Tube No 16 3 ml per tube

pH 5 51 Elution was performed with 0 2 M phosphate buffer of pH 6 90 (Fig 8) it was found that the bulk of the ICSH activity was eluted from Tubes 80–85 The contents of these tubes were then combined dialysed and lyophilized The lyophilized product (17 mg) designated fraction HC was purified human ICSH which then was subjected to various analyses for biological and physicochemical characterization

Table VIII summarizes the yield and estimated ICSH potency of fractions obtained from each step in the purification

in which A is the ovarian weight in mg and D the total dose in micrograms

When FSH fraction H was injected into hypophysectomized rats, it was apparent that it was not completely free from contamination with ICSH, as evidenced by its effect on uterine weight in female rats and on ventral prostate weight in male rats From the latter data, the estimated amount of contaminating ICSH activity present in fraction H is of the order of 11 per cent This estimate rests upon two assumptions, that human FSH *does not possess any intrinsic interstitial* cell stimulating activity, and that the biological activity of pure ICSH does not differ in any significant aspect from that of the highly purified hormone, fraction HC, whose preparation is described below

Purification and properties of human ICSH (With P G Squire)

When at one step of the isolation of growth hormone from human pituitaries an ammonium sulphate precipitate was submitted to chromatography on an Amberlite IRC 50 resin *column the hypophysial ICSH activity* was found to* reside in the material that passsed through the column unadsorbed From an original 10 g of lyophilized human pituitary glands 150 mg of material were obtained, this material was designated fraction U (see Fig 6) Fraction U was further purified as follows a 0 3 per cent solution of the fraction was carefully adjusted to pH 4 5 with 0 1 M HCl The resulting precipitate containing little activity was removed by centrifugation The clear supernatant fluid was brought to 2 0 M $(NH_4)_2SO_4$ by the addition of an equal volume of saturated ammonium sulphate solution The precipitate that formed was dissolved dialysed and lyophilized From 150 mg of fraction U, 95 mg of purified ICSH fraction IIA were obtained Fraction IIA was

* The interstitial-cell stimulating activity of the various fractions was assayed by the ventral prostate test (Greep Van Dyke and Chow 1941) in rats of the Long Evans strain (Lostroh Squire and Li 1958) A unit of ICSH is defined *as the amount of ICSH that will elicit a mean ventral prostate weight* double that of a control group

HC indicating the possible presence of contaminating FSH activity This was confirmed by the augmentation test (Steelman and Pohley 1953), whereby 0 10 mg of fraction HC was administered together with human chorionic gonado tropin (HCG) to 21 day old female rats of the Long Evans strain With this dose of purified ICSH an increment in ovarian weight was observed, amounting to 15 9 mg over the weight of the ovaries of the HCG treated controls A total dose of 0 2 mg of fraction HC injected for a period of 4 days by the subcutaneous route produced no histological changes in the adrenals and thyroids of hypophysectomized immature female rats nor did it have any effect on the width of the tibial epiphysial cartilage plate These data suggest that the ICSH fraction HC is practically free of thyrotropic adrenocor ticotropic and growth hormone activities

FSH fraction H and ICSH fraction HC were both subjected to preliminary ultracentrifugational studies The sedimenta tion pattern of ICSH showed the presence of a major compo nent (amounting to 82 per cent) with a sedimentation co efficient ($S_{20,w}$) of 2 14 S and a minor component with a higher sedimentation coefficient The sedimentation pattern of FSH was more complex but if it is analysed as if it were a two component system the major component (70 per cent) possesses a sedimentation coefficient of 1 67 S At least in the case of the ICSH it is entirely possible that the rapidly sedimenting component may arise through the formation of polymers from monomeric units

Comparison of human gonadotropins with the ovine hormones

The leak points i e the respective characteristic pH values below which the ICSH activity is adsorbed on to Amberlite IRC 50 resin above which it passes through the column unadsorbed and at or very near which the protein leaks off as a peak followed by tailing are distinctly different for ICSH preparations from sheep and from human pituitar ies in 0 2 M phosphate buffer the 'leak point' of the ovine

Table VIII

YIELD AND POTENCY OF FRACTIONS OBTAINED FROM PURIFICATION PROCEDURE OF HUMAN ICSH

Fraction	*Yield*	*Procedure*	*Activity*
	mg		units/mg
A	10 000	Lyophilized pituitaries	
U	150	IRC-50 resin unadsorbed obtained from growth hormone isolation steps	37
HA	95	pH 4 5 $(NH_4)_2SO_4$ fractionation	57
HB	33	Column electrophoresis	135
HC	5	IRC-50 resin chromatography	240

Five milligrams of purified ICSH were obtained from 10 g of lyophilized whole human pituitaries The effect of this purified ICSH fraction on ventral prostate weight in hypophysectomized rats summarized in Table IX, can be expressed by the equation

$$P = 24\ 8 \log D + 0\ 6$$

where D is the dose in milligrams Thus a dose of 3 9 µg of fraction HC will elicit an increment in ventral prostate weight of 100 per cent over the controls

It can be noted from Table IX that the weight of the testes was increased significantly by a total dose of 12 µg of fraction

Table IX

BIOLOGICAL ACTIVITY OF ICSH FRACTIONS HC IN HYPOPHYSECTOMIZED MALE RATS

Total dose	*No of rats*	*Ventral prostate*	*Testes*
µg		mg	mg
0	8	7 7 ± 2 1*	135 ± 5
3	5	12 4 ± 1 4	149 ± 8
4	20	16 4 ± 0 7	
8	8	22 6 ± 2 4	
12	6	27 8 ± 5 2	258 ± 11
20	7	36 3 ± 4 0	

* Mean ± S.E.

tion SH when injected intramuscularly for a few days in pre menopausal women on the day of cessation of menstruation resulted in the development of multiple follicular cysts in the ovary The clinical effectiveness of human ICSH has also been demonstrated (Bergenstal, 1958–9)

Summary

Biological immunochemical and physicochemical investigations on human pituitary growth hormone, which have recently been carried out in this laboratory, are summarized and discussed In addition from the side fractions procured in the course of the isolation of human pituitary growth hormone it is reported that it is now possible to prepare human follicle stimulating hormone (FSH) and human inter stitial cell stimulating hormone (ICSH) in highly purified form Physicochemical and biological studies of these two gonadotropins derived from human pituitary glands have revealed differences between them and the preparations ob tained from sheep pituitaries

Acknowledgments

The author wishes to express his indebtedness to Drs Ute Groschel Tetsuo Hayashida Ardis J Lostroh Raghu Moudgal Harold Papkoff and Phil G Squire for permission to present unpublished data Grateful acknowledgment is made to Frank Fearney Charles W Jordan Jr and Richard L Wilcox for their able technical assistance The work was supported in part by grants from the National Institutes of Health of the United States Public Health Service (RG 4097) the American Cancer Society the Albert D and Mary Lasker Foundation the Upjohn Company Eli Lilly and Company and the Cutter Laboratories

REFERENCES

BATES R W and CORNFIELD J (1955) *J clin Endocr* 15 838
BERGENSTAL D (1958–9) Personal communications
CHOW B F (1942) *Endocrinology* 30 657
FLODIN P and KUPKE D W (1956) *Biochim biophys Acta* 21, 868
GEMZELL C A and LI C H (1958) *J clin Endocr* 18 1
GREENSPAN F S LI C H SIMPSON M E and EVANS))
Endocrinology 45 455
GREEP R O VAN DYKE H B and CHOW B F
exp Biol (N Y) 46 644

hormone is located at pH 6 2 and that of the human hormone at pH 5 6 Human ICSH fraction HC also behaved quite differently from the sheep hormone in electrophoresis on starch (Squire and Li, 1959) According to the pattern obtained from electrophoresis of ovine and human ICSH preparations in 0 103 M carbonate buffer of pH 8 6, the human ICSH is more negatively charged than is the ICSH from sheep glands

With respect to biological action the human ICSH appears to be more effective in promoting increase in ventral prostate in hypophysectomized rats The slope of dose to response calculated for the human ICSH fraction HC is more than three times that obtained for the ovine hormone (Lostroh Squire and Li 1958, Squire, 1957) It is unlikely that these differences can be attributed to the estimated 2 per cent contamination by FSH in the human ICSH preparation Table X summarizes some properties of the ICSH preparations from the two species

Table X

COMPARISON OF HUMAN AND SHEEP ICSH

Properties	ICSH	
	Human	*Sheep*
Leak point*	pH 5 6	pH 6 2
Sedimentation coefficient ($S_{20\,w}$)	2 14 S	2 47 S
Isoelectric point	≤ pH 7 3	pH 7 3
Dose response equation†	$I = 24\ 8 \log D + 0\ 6$	$P = 7\ 2 \log D + 12\ 1$

* Amberlite IRC 50 r in equilibrated with 0 2 M potassium phosphate buffer
† Mal r ts w re hypophysectomized at 1 days of ag an l ul uta eo s l jecti n begi days post perati e on e d ll for 4 day autopsied 4 h urs aft r t e l t l jecti I = v ntral prostate in mg D = dose g in µg

Although no actual comparisons have been made between the ovine and human FSH preparations it can probably be expected that they are not identical proteins In clinical investigations ovine FSH preparations are not potent in producing follicle stimulation in young female subjects whereas the human hormone is extremely active at low dosages (Bergenstal 1958–9) in fact as low a daily dose as 1 mg of FSH frac

tivity of the animals used? The fact that a standard preparation is not being employed means that the results obtained by the two laboratories cannot be compared directly

Li That is an interesting point The Sprague Dawley strain is more sensitive than the Long Evans strain with respect to ventral prostate weight as influenced by ICSH

Sonenberg I have been interested in some further differences between bovine and human growth hormone in certain respects you have reported that bovine growth hormone cannot be reduced by a whole series of procedures Has this been tried with human growth hormone with the two disulphide links?

Li No

Sonenberg One could possibly also get some information about secondary structure It has been reported that urea will not de nature growth hormone on the basis of biological activity Has the same thing been tried with human growth hormone?

Li The problem is that you cannot inject the urea solution into the rat because of its high toxicity You have to dialyse out the urea for your assays and although the urea itself may change the secondary or tertiary structure removal by dialysis will cause the structure to return to its original form For example in the case of beef growth hormone it takes about 10 M urea to increase the optical rotation from −41 to the maximal −96 but when you dialyse out the urea the optical rotation returns to −41 Thus it has not been possible to study the effect of secondary structure on activity

Sonenberg Then you have been able to get reduction of bovine growth hormone?

Li Yes in the presence of 10 M urea

Sonenberg We have had a different experience We have been able to inject 8 M urea with the growth hormone into hypophy sectomized Sprague Dawley animals and have had assays identical with those obtained with the use of non urea treated growth hor mone The animals seem to survive the procedure This was on the basis of a weight gain test

Have you any immunological evidence that the chymotrypsin treated bovine growth hormone will respond to anything prepared in response to human growth hormone?

Li That is an interesting question The procedures for the pro duction of rabbit antiserum to beef growth hormone and human growth hormone have both been published (Hayashida and Li 1958*a* and *b* *loc cit*) In order to get a good antiserum to beef growth hormone you need a total dose of about 80 mg whereas in the case of the human growth hormone you need only 8 mg We take the

HAYASHIDA T and LI C H (1958*a*) *Endocrinology* 63 487
HAYASHIDA T and LI C H (1958*b*) *Science* 128 1276
HAYASHIDA T and LI C H (1959) *Endocrinology* 65 944
HOREJSI J and SMETANA R (1956) *Acta med scand* 155 65
LI C H (1957) *Fed Proc* 16 775
LI C H (1958) *Proc Soc exp Biol (N Y)* 98 839
LI C H LYONS W R and EVANS H M (1940) *J biol Chem* 136 709
LI, C H, LYONS W R and EVANS H M (1941) *J gen Physiol* 24 303
LI C H and PAPKOFF H (1956) *Science* 124, 1293
LI C H PAPKOFF H and JORDAN C W Jr (1959) *Proc Soc exp Biol (N Y)* 100 44
LOSTROH, A J and LI C H (1957*a*) *Endocrinology* 60 308
LOSTROH, A J and LI C H (1957*b*) Unpublished data
LOSTROH A J SQUIRE P G and LI C H (1958) *Endocrinology* 62 833
PORATH J (1956) *Biochim biophys Acta* 22 151
SQUIRE P G (1957) Thesis University of California
SQUIRE P G and LI C H (1959) *J biol Chem* 234 520
STEELMAN S L and POHLEY F M (1953) *Endocrinology* 53 604
STETTEN D (1956) *Science* 124 365
VAN DYKE H B PAN S Y and SHEDLOVSKY T (1950) *Endocrinology* 46 563

DISCUSSION

Wilhelmi Prof Li have you made any observations as to the distribution of TSH in your fractions ?

Li We have had a very difficult time locating the TSH Apparently the TSH content is very low As you have estimated it in your report to this colloquium it is about 1 unit/g Thus, in order to isolate any TSH from extracts one would have to have a very fair amount of material to start with

Wilhelmi That is our impression too From the experiments that we have tried it appears that either the TSH or FSH comes out much better if one extracts on the acid side I think the difference in the calculation of your FSH yields and ours is that we are getting about seven times as much We translated our own figures into slope units and I think from data which you reported a while ago that you get approximately one million slope units/kg (Li 1958 *loc cit*) by our estimate it is about seven million slope units/kg

Li So you suggest that the calcium oxide insoluble fraction might be recoverable

Wilhelmi It could be

Loraine Prof Li could the discrepancy between results obtained by you and Prof Wilhelmi be due merely to variations in the sensi

tivity of the animals used? The fact that a standard preparation is not being employed means that the results obtained by the two laboratories cannot be compared directly

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Li So you suggest that the calcium oxide-insoluble fraction might be recoverable

Wilhelmi It could be

Loraine Prof Li could the discrepancy between results obtained by you and Prof Wilhelmi be due merely to variations in the sensi

Li No we have not The amount of insoluble material is really very small We used the whole suspension for the experiments with the tibia test

Beck You said you required as much as 80 mg of bovine growth hormone to develop a sufficiently high titre antiserum What is the method of this administration?

Li By both subcutaneous and intraperitoneal injection

Beck Drs Fishman McGarry and I have been interested in this point too (Fishman J B McGarry E E and Beck J C (1959) *Proc Soc exp Biol* (*N Y*) **102** 446) We are using a different preparation of growth hormone and can achieve good antibody titres We have switched from the intramuscular to the intradermal route

Wilhelmi One further comment with respect to resistance in your experiments Prof Li during the development of resistance injections were continuous In our experience if we have e g a set of rats which have been given monkey or human growth hormone for just ten days and then are allowed to rest for two weeks and are given again monkey or human growth hormone they are still resistant so that continuous injection is unnecessary Resistance develops during the interval and it stays But if one resumes with sheep horse pig or bovine growth hormone the response is perfectly good

Beck How long does this resistance persist?

Wilhelmi That is hard to say For reasons of economy we use our hypophysectomized rats twice and they are allowed two weeks rest from the end of one 10 day assay to the next this is the longest period during which we can state that the resistance is there

antiserum to beef growth hormone and allow it to react with the *beef hormone* measuring the reaction by either the Ouchterlony agar gel method (Ouchterlony O (1953) *Acta path microbiol scand* 32 231) or by the precipitin ring test The chymotrypsin digest which is biologically active material does not react with the *antiserum to beef growth hormone* Furthermore the antiserum to human growth hormone again does not precipitate with the chymotrypsin treated beef growth hormone

Sonenberg Have the positive results on the reduction of the di sulphide links in growth hormone been *published?*

Li No the procedure is the same as in the lactogenic hormone reduction (Li C H and Cummins J T (1958) *J biol Chem* 233 73) You can reduce the disulphide bridges by means of mercaptoethanol in 10 M urea

Sonenberg What about the biological activity ?

Li The biological activity is lost We have tested only the beef but not the human hormone

Raben Dr Gemzell do you still believe that some pituitary glands have no growth hormone ?

Gemzell Every month pituitaries are collected from three different hospitals in Stockholm I usually purify the growth hormone from each bunch separately and find constantly the same difference in yield the pituitaries are always prepared and frozen in the same way yet from time to time there is a constant difference The only way I can explain this difference is that the three hospitals treat *different kinds of patients*

In order to find out if there is any connexion between the amount of growth hormone stored in the pituitary and the cause of death of the patient I have tried to purify growth hormone quantitatively *from a single pituitary (Gemzell and Li 1958 loc cit)* One striking fact was that patients who had been operated upon shortly before death had a very low growth hormone content I have examined the pituitaries of two patients who died of severe burns and the growth hormone content was also low Very little difference from normal levels (4–5 mg /pituitary) was found in the pituitaries of patients who had died of other causes

I have also estimated the concentration of growth hormone in the plasma of patients following surgical operation (Gemzell C A (1959) *J clin Endocr* 19 1049) Six hours after operation the concentration was elevated I would suggest that there exists some correlation between the low content of growth hormone in the pituitary and the elevated plasma level

Beck Prof Li have you assayed the precipitate *from your saline* extract of single pituitaries in the tibia test ?

to growth hormone by a modification of Boyden s haemag glutination method (Boyden, 1951) Thrice saline washed sheep erythrocytes were exposed to a dilute solution of tannic acid in pH 7 2 buffered isotonic saline The tannic acid coated cells were washed and suspended in isotonic buffered saline at pH 6 4 Human growth hormone in a concentration of 1 0 mg /ml was diluted to 6 0 ml with pH 6 4 isotonic buffered saline This solution was added in equal quantities to the cell suspension After incubation at 37 the growth hormone coated tanned cells were washed with normal guinea pig serum diluted 1 80 in isotonic saline and sus pended in that medium at a concentration of 2 8 per cent

These treated cells then were added to doubling dilutions of rabbit serum and incubated at room temperature After three hours the appearance of the cells in the bottom of the test tube was examined When an antibody to the growth hormone was present haemagglutination of the cells occurred Under these circumstances the cells were distributed over the bottom of the test tube forming an even umbrella With very strong agglutination the sheet of cells was sometimes dis torted and wrinkled A minor degree of haemagglutination resulted in a circle of cells surrounding a central film With no haemagglutination a discrete button or bull s eye was formed

The results of testing the pre immunization and post immunization sera as well as the normal guinea pig serum used as a diluent for the presence of an antibody are shown in Table I No haemagglutination was seen following the addition of washed sheep cells or tanned cells to any of the three sera or when the growth hormone treated cells were added to the pre immunization or normal guinea pig sera However when the growth hormone cells were added to the post immunization serum complete haemagglutination was seen even when the serum was diluted to 1 1 280 The next dilution 1 2 560 showed only a minor degree of haemag glutination and there was none at a dilution of 1 5 120 The titre of the antiserum defined as the greatest dilution of the

IMMUNOLOGICAL STUDIES OF HUMAN GROWTH HORMONE*

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THE purpose of this paper is to record immunological studies of human growth hormone carried out in the course of developing a method for assaying this hormone in human serum

To make the antigen human growth hormone (Raben) was dissolved in water in a concentration of 1 0 mg /ml with the aid of a few drops of 0 1 N sodium hydroxide and mixed with an equal volume of a mineral oil and lanolin adjuvant (Ramon Lemetayer and Richou 1935) Two millilitres of this mixture were injected subcutaneously into adult female rabbits at weekly intervals for a total of three injections Serum obtained before the first injection was tested to make sure that it did not contain a false haemagglutinin One week after the third injection the rabbit serum was tested for the presence of an antibody If a low titre was found 1 0 mg of growth hormone diluted to a 10 ml volume in isotonic saline was injected intravenously One week to ten days later the titre was rechecked and if it was more than 1 1 200 40–50 ml of blood were withdrawn After six or more weeks a second dose of growth hormone was given intravenously This time the animal was exsanguinated when the titre reached a satisfactory level usually in five to ten days This limitation of the number of injections of growth hormone was to minimize the possibility of stimulating the production of antibodies to possible contaminants of the growth hormone

The rabbit serum was tested for the presence of an antibody

* Supported by a grant from the College of Medicine Trust Fund State University of Iowa

serum which resulted in complete haemagglutination, was thus 1 1,280

The ability of the antigen growth hormone, to inhibit the anti serum *in vitro* was demonstrated by using a haemagglutination inhibition technique (Boyden 1951) The principle of this procedure is that when to the antiserum which in its final concentration is equal to or more concentrated than its titre no growth hormone is added free antibody remains if growth hormone cells are then added haemagglutination of these cells occurs However if a large amount of growth hormone is added to the antiserum all of the antibodies are neutralized, when growth hormone cells are added subsequently no haemagglutination occurs When decreasing concentrations of growth hormone are added to successive tubes containing the same concentration of antiserum eventually a point is reached where not all of the antibodies are neutralized free antibody remains and causes haemagglutination of the growth hormone cells At first such haemagglutination is weak but complete haemagglutination is seen in the tubes containing sufficiently small concentrations of growth hormone The end point is that concentration of growth hormone at which complete (4+) haemagglutination first occurs

The results of a haemagglutination inhibition test are presented in Table II Decreasing amounts of growth hormone

Table II

INHIBITION OF GH ANTI SERUM (RB) BY GH

	m µg /ml GH													
	0 0	400	300	200	100	80	60	40	20	10	8	6	4	1
1 1 400 rb AB	4	0	0	0	2	3	3	3	4	4	4	4	4	4
Pre inject rb	0	0	0	0	0	0	0	0	0	0	0	0	0	0

(GH) were added in successive tubes to an antiserum with a titre of 1 1 400 In those tubes in which the concentration

Table I

RESULT OF TESTING PRE AND POST IMMUNIZATION RABBIT SERA FOR AN ANTIBODY TO HUMAN GROWTH HORMONE

Cell preparation	*Dilutions of serum*									
	1 10	1 20	1 40	1 80	1 160	1 320	1 640	1 1 280	1 2 560	1 5 120
					Pre immunization serum (rb)					
SP	0	0	0	0	0	0	0	0	0	0
T SP	0	0	0	0	0	0	0	0	0	0
GH T SP	0	0	0	0	0	0	0	0	0	0
					Post immunization serum (rb)					
SE	0	0	0	0	0	0	0	0	0	0
T SE	0	0	0	0	0	0	0	0	0	0
GH T SE	4	4	4	4	4	4	4	4	1	0
					Diluent normal serum (gp)					
SP	0	0	0	0	0	0	0	0	0	0
T SP	0	0	0	0	0	0	0	0	0	0
GH T SP	0	0	0	0	0	0	0	0	0	0

rb rabbit gp guinea pig SF sheep erythrocyte T SE tanned sheep erythrocyte GH T SF growth hormone treated tanned sheep erythrocyte

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Pre inject rb	0	0	0	0	0	0	0	0	0	0	0	0	0	0

(GH) were added in successive tubes to an antiserum with a titre of 1 1 400 In those tubes in which the concentration

of growth hormone was 400 300 and 200 m µg /ml respectively the antiserum was completely neutralized A minor degree of haemagglutination was observed first in the 100 m µg growth hormone tube Complete haemagglutination (4+) was noted first in that tube to which growth hormone in a concentration of 20 m µg /ml had been added The pre injection rabbit serum was inactive

The presence in the rabbit serum of an antibody to human growth hormone also was demonstrated by other serological methods Using the complement fixation method the titre of the antiserum was 1 64 it was anti complementary to a dilution of 1 24 The appearance of a precipitin in the single and double gel diffusion studies to be described below indicated that the antiserum was demonstrable by this third technique Other studies to be presented elsewhere have provided further confirmation (Read and Bryan 1960) an anamnestic response has been observed and the well known biological effect of growth hormone on the tibial plate of the hypophysectomized rat has been inhibited by the antiserum whether the growth hormone and antiserum were mixed prior to injection or whether they were injected by separate routes

Human serum was tested to find out if it was able to inhibit the activity of the antiserum The sera of three normal adults and three normal children as well as three patients who had been subjected to hypophysectomy and one child with hypopituitarism were so examined Dilutions of the sera were made with 1 80 normal guinea pig serum and added to an equal volume of the antiserum After addition of the growth hormone cells the mixture was incubated at 4° for three hours The results are reported in Table III All of the sera of the normal children and adults inhibited the antiserum In contrast no inhibition of the antiserum was observed when the sera of two of the hypophysectomized patients or of the child with hypopituitarism were added Only a slight inhibition was caused by the serum of one hypophysectomized patient This patient subsequently died and at autopsy was found to have remaining some pituitary tissue These

Table III

INHIBITION OF GH ANTI SERUM (RB) BY HUMAN SERUM

Source of serum		*Dilutions of human serum*										
		1:2	1:4	1:8	1:16	1:24	1:32	1:48	1:64	1:96	1:128	1:192
Normal	adult ♂	0	0	0	2	3	4	4	4	4	4	4
	adult ♂	0	0	0	3	4	4	4	4	4	4	4
	adult ♀	0	0	3	3	4	4	4	4	4	4	4
Normal	5 yr ♀	0	0	0	4	4	4	4	4	4	4	4
	4 yr ♂	0	0	3	4	4	4	4	4	4	4	4
	8 yr ♀	0	0	0	3	4	4	4	4	4	4	4
Hypophysectomized	adult ♀*	0	3	4	4	4	4	4	4	4	4	4
	adult ♂	4	4	4	4	4	4	4	4	4	4	4
	adult ♂	4	4	4	4	4	4	4	4	4	4	4
Hypopituitarism	6 yr ♂	4	4	4	4	4	4	4	4	4	4	4

* Incomplete hypophysectomy

findings showed that not only was there an inhibiting factor in human serum, but also that it was of pituitary origin

Both haemagglutination and inhibition of haemagglutination procedures were used to examine the reaction between the growth hormone antiserum and a number of human proteins Gamma globulin serum albumin and lyophilized whole serum were reconstituted and incubated with the tanned cells in the same concentrations previously used for growth hormone Haemagglutination reactions were carried out also with human growth hormone prepared by Wilhelmi and by Li as well as by Raben The antiserum did not haemagglutinate gamma globulin albumin or whole serum treated cells It did haemagglutinate the cells which had been incubated with any of the growth hormone preparations Similarly the gamma globulin and albumin even in concentrations as high as 0 5 mg /ml did not inhibit the antiserum It was inhibited by the human serum and by all three growth hormone preparations

The antiserum to human growth hormone was tested with a number of growth hormone preparations of other species as well as other protein hormones of non human origin In each instance both haemagglutination and inhibition tests were done the latter at a sensitivity sufficient to detect concentrations as low as 0 004 μg /ml The results presented in Table IV showed that there was no reaction between the human growth hormone antiserum and insulin (either monkey or beef) or commercially available beef thyroid stimulating hormone (TSH) or hog adrenocorticotropic hormone (ACTH) Similarly growth hormone made from beef hog or horse pituitaries did not react with the antiserum Monkey growth hormone made by either Raben or Wilhelmi reacted with human growth hormone antiserum both in the haemagglutination and haemagglutination inhibition procedures With fish growth hormone there was a minor degree of interaction in the inhibition test This was interpreted as meaning that fish and human growth hormone have in common an immunologically active group The results obtained in this experiment indicate

Table IV

REACTIONS BETWEEN HUMAN GH (RABEN) ANTISERUM (RB) AND PROTEIN HORMONES OF OTHER SPECIES

Hormone	*Source*	*Haemagglutina tion*	*Haemag – Inhibition*
Insulin	monkey—Lilly	No	No
Insulin	beef—Lilly T3202	No	No
TSH	beef—commercial	No	No
ACTH	hog—commercial	No	No
GH	beef—Armour—22KR2	No	No
GH	beef—Wilhelmi—B168	No	No
GH	hog—Wilhelmi—P214A	No	No
GH	horse—Wilhelmi—H264C	No	No
GH	fish—Wilhelmi—F80	No	Slight
GH	monkey—Wilhelmi—N334 B41B	Yes	Yes
GH	monkey—Raben	Yes	Yes

that those components of the growth hormone molecule which determine immunological specificity are similar in primates and are different from those of lower animals Similar results have been obtained by Hayashida and Li (1958)

The results of the experiments described thus far made it probable that the growth hormone antiserum was specific at least for proteins of pituitary origin The next series of experiments was done to find out whether the antiserum was specific for growth hormone or whether it would react with other proteins of the human pituitary The human pituitary proteins used were made by Dr Raben in the course of the preparation of growth hormone (Raben 1957) The fractions were acetone precipitate ACTH pH 8 5 precipitate and lyophilized supernatant They were dissolved in water with the aid of 0 1 N sodium hydroxide the stock concentrations being 1 0 mg /ml For weaker concentrations dilutions were made with normal guinea pig serum diluted 1 80 in isotonic saline

The antiserum was examined first by the haemagglutination technique the tannic acid treated cells being incubated with each fraction in the same concentration used for preparing growth hormone treated cells The antiserum caused haemagglutination of all four kinds of protein treated cells

however, in the case of ACTH haemagglutination occurred in some experiments and not in others The antiserum was examined for lack of homogeneity also by the method of Preer (1956) In this technique the antiserum and the antigen are interspaced by a neutral gel layer and both diffuse into the gel The double diffusion methods enjoy certain advantages over the single diffusion techniques, for the relative concentrations of the antigen and antibody are not so critical and because the location of the lines is dependent on the diffusion coefficients of both antigen and antibody, overlapping of bands is less likely Additionally the bands may be spread by increasing the length of the gel column (Augustin and Hayward 1955) The Preer method also allows the use of undiluted antiserum and yet because of the micro tubes used is very saving of materials Furthermore, two antigens each of which shows precipitation zones with the same antiserum can be separated easily by mutual dilutions of both substances being allowed to diffuse against the antiserum If the precipitable antigens are identical the mixture shows but one precipitation zone

The antigens used in the first experiment were growth hormone supernatant pH 8 5 precipitate ACTH and acetone precipitate The antiserum was used undiluted The tubes were photographed after three days incubation at room temperature In Fig 1 the antigen was growth hormone in concentrations of from left to right 100 90 75 50 25 10 and 0 μg /ml In those tubes with the highest concentrations of growth hormone the antigen was present in excess the zone of precipitation being seen only as a broad band beginning at the antiserum-gel interface At a concentration of 50 μg /ml the precipitation zone was barely above the interface but at 10 μg /ml it was seen as a clear line in the middle of the gel In no instance was there evidence of more than one band

Lesser concentrations of growth hormone were used in the series of tubes shown in Fig 2 They were from left to right 10 9 7 5 5 2 5 1 0 and 0 μg /ml As the concentration

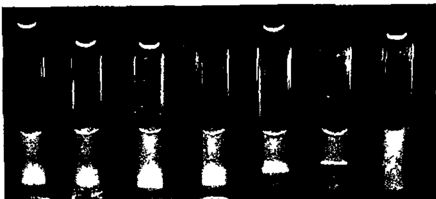


FIG 1 Growth hormone (high concentrations)

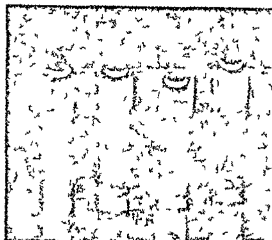


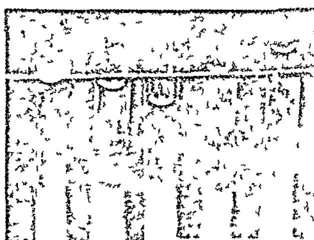
FIG 2 Growth hormone (low concentrations)



FIG 3 Supernatant

facing page 6





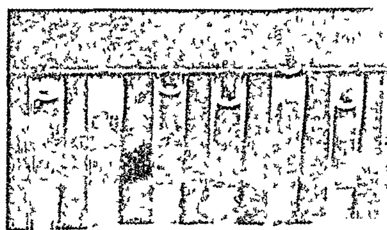
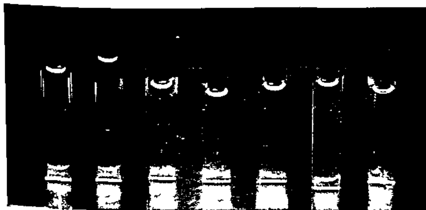





Fig 7 Mutual dilutions of growth hormone and supernatant



Fig 8 Mutual dilutions of growth hormone and pH 8 5 precipitate



ig 9 Mutual dilutions of growth hormone and ACTH



FIG 10 Mutual dilutions of growth hormone and acetone precipitate



FIG 11 Crude pituitary extract (the light areas above the antigen gel interface are photographic artifacts)

FIG 12 Mutual dilutions of crude pituitary extract and

decreased the precipitation line approached the antigen-gel interface Although a band was barely discernible in the tube containing growth hormone in the concentration of 2 5µg /ml none was seen in the 1 5 µg /ml tube nor in the control tube

The next series of photographs Figs 3 4 5 and 6 show the lines formed when supernatant pH 8 5 precipitate ACTH and acetone precipitate were similarly tested The concentrations with each antigen were 100 90 75 50 25 10 and 0 µg /ml reading from left to right Single bands were seen in the higher concentration of all four antigens indicating that each reacted with the antiserum

To determine whether these bands were due to one or more antigens mutual dilutions of growth hormone and each pituitary fraction were made and allowed to diffuse against the antiserum Thus from left to right the tubes shown in Figs 7 8 9 and 10 contained (µg /ml) the following growth hormone 10 growth hormone 9 and pituitary fraction 10 growth hormone 7 5 and pituitary fraction 25 growth hormone 5 0 and pituitary fraction 50 growth hormone 2 5 and pituitary fraction 75 growth hormone 1 0 and pituitary fraction 90 and pituitary fraction 100 respectively In every instance there was but one precipitation line formed by growth hormone and the pituitary fraction with which it was mixed namely supernatant pH 8 5 ACTH and acetone precipitate Thus there was no evidence of the presence of more than one antibody

Although it was believed that all of the pituitary hormones were present in one or more of the fractions tested an additional experiment was carried out using the Preer method in which the antigen was a crude saline—2 per cent butanol extract of human pituitary gland The length of the agar column was increased to spread apart any lines that might be formed Fig 11 shows that the crude pituitary extract gave only one band of precipitation with the antiserum When the pituitary extract was mutually diluted with growth hormone again only one line was seen (Fig 12)

The growth hormone antiserum was also examined by adsorption tests and by single agar diffusion (Oudin 1952) and by these methods also no evidence of lack of homogeneity was obtained (Read and Bryan 1960) It seemed that the reaction between the antiserum and the pituitary fractions was due to their growth hormone content rather than to a non specific reaction between the antiserum and pituitary proteins in general Whatever contaminants there might be in the Raben growth hormone preparation were not present in sufficient quantities to result in antibody formation by the immunization procedure described above and/or detectable by the methods used

In the assay of growth hormone in human serum the inhibition of the antiserum by known concentrations of growth hormone and by unknown concentrations in human serum was carried out simultaneously The unknown sera and the growth hormone standards were set up in quadruplicate at the same antibody concentration When a very sensitive assay was required the dilution of the antiserum used in the inhibition tests was the same as the predetermined titre Ordinarily the antiserum was used in a lesser dilution than the titre for the difficulty of reading the end point was decreased and the number of dilutions of the test sera being assayed was reduced For example if the antiserum titre was 1 1 400 and was used in this dilution in the inhibition the first completely haemagglutinated tube was likely to be either the 2 or 4 m µg /ml standard However if a dilution of 1 1 000 was used the end point would then likely be in the 15 or 20 m µg /ml standard The method has been described in detail elsewhere (Read 1960)

The statistical process of estimating the potency of the unknown and assessing the fiducial limits of the individual assay is simple This is so because the response is complete haemagglutination and the end point is that concentration of growth hormone or test serum in which this just occurs Thus the threshold dose is a dependent variable and is determined at any single antibody concentration by dilution of the antigen

This is comparable to the "cat" method for the assay of digitalis in which the end point is the death of the cat. It has the advantage however that the size of the cat can be rigorously controlled since the same dilution of antiserum is used both in the standards and unknowns. In such direct assays the relative potency is obtained as the difference between the mean logarithms of the concentration of the growth hormone in the standard and the dilution of the test serum in the first tubes completely haemagglutinated. The statistical procedure as well as the method of determination of the 95 per cent confidence limits has been fully discussed by Finney (1952).

The results obtained in studying the homogeneity of the antiserum by absorption, single gel diffusion and double gel diffusion methods indicated that the pituitary protein fractions contain growth hormone. In order to substantiate this conclusion the pituitary fractions were assayed for their growth hormone content by both physiological and immunological methods. The bioassay method used was that of Greenspan and co-workers (1949). A preliminary experiment showed that in the dose range of 25 to 400 µg of human growth hormone (Raben) there was a linear relationship between the logarithm of the dose and the width of the tibial plate. Four point parallel line assays were done by injecting a high and a low dose of the human growth hormone standard and each of the pituitary fractions into appropriate groups of rats. Because the regression lines resulting from injecting ACTH and pH 8.5 were found to be not parallel to the regression line of growth hormone it was impossible to determine the relative potency of these two fractions by the bioassay method. However the regression lines of the supernatant and acetone precipitate were not different from parallelism and relative potencies were derived for these pituitary fractions. In doing so the assumption was made that growth hormone was the only substance present which affected the width of the tibial plate and to the extent that this may not be true the assays may be unreliable.

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The relative growth hormone potencies of acetone precipitate and supernatant as measured by the two assay methods are presented in Table V By the tibial plate method acetone

Table V

A COMPARISON OF RELATIVE GH POTENCIES OF ACETONE PRECIPITATE AND SUPERNATANT AS MEASURED BY PHYSIOLOGICAL AND IMMUNOLOGICAL METHODS

(GH Standard—Raben Homo No 6)

	Assay method	
	Tibial plate	Immunological
Acetone precipitate	17 / (11–31)	24 / (11–55)
Supernatant	48 / (44–51)	21 / (9–51)

Figures in parentheses are 95 / confidence limits

precipitate was 17 per cent as potent as the growth hormone standard whereas by the immunological method its potency was 24 per cent A greater difference was noted in the results of the assays of supernatant for by the bioassay method it had 48 per cent of the potency of growth hormone whereas by the immunological method it was but 21 per cent as potent However these potencies cannot be considered as significantly different since the assay values obtained by the bioassay are within the 95 per cent confidence limits of the immunological method

The ACTH and pH 8 5 precipitate fractions were assayed by the immunological method Their relative potencies were found to be 3 per cent and 9 per cent respectively of the growth hormone standard as is recorded in Table VI Two other fractions were assayed also A second acetone precipitate was made by Dr Raben by redissolving the original acetone precipitate and attempting to remove from it more growth hormone In a comparable fashion Dr Raben made a second supernatant by redissolving the first supernatant and repeating the procedure for precipitating the growth hormone In

Table VI

RELATIVE GH POTENCIES OF PITUITARY PROTEIN FRACTIONS (HOMO NO 6)

(GH Standard—Raben Homo No 6)

Fraction	*Percentage of standard*	*95 per cent confidence limits*
Acetone precipitate	24	11–55
Second acetone precipitate	1 07	0 8–1 36
ACTH	3	—
pH 8 5	9	4–21
Supernatant	21	9–51
Second supernatant	1 6	1 2–2 1

both instances these procedures resulted in a great decrease in the amount of growth hormone in the original fraction as determined by the assay The relative potency of the second acetone precipitate was but 1 07 per cent which compared with 24 per cent in the first acetone precipitate and the relative potency of the cleaned supernatant was but 1 6 per cent which contrasted with 21 per cent of the original supernatant

Serum growth hormone assays of a small number of normal children and adults as well as patients with acromegaly and hypopituitarism have been made to date using as the growth hormone standard Raben s Homo No 6 As is shown in Fig 13 the growth hormone levels in 14 normal children were between 90 and 600 m μg /ml However in none of the nine hypopituitary patients were the levels over 90 m μg /ml In contrast all of the levels in nine patients with apparently active acromegaly were above 500 m μg /ml Thus, in acromegaly high values were found and in hypopituitarism low values were observed Further assays are now being carried out to determine normal levels in adults and in children

In collaboration with Dr Raben an experiment was carried out in which each of three patients suffering from hypopituitarism was injected with 4 mg of growth hormone In two of these patients the growth hormone was given intramuscularly in the third it was given intravenously Blood was drawn before and at variable intervals after the injections The

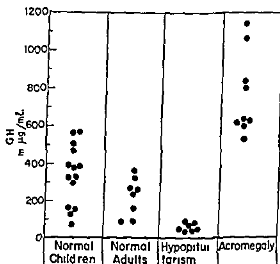


FIG 13 Growth hormone levels of human serum Not shown two hypopituitary patients in whom there was no detectable growth hormone

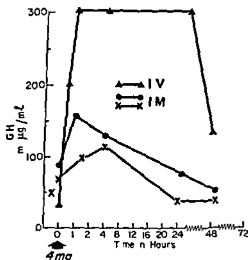


FIG 14 Effect of injecting human growth hormone on the serum growth hormone levels of three hypopituitary patients

serum was separated and kept frozen until assayed As is shown in Fig 14 the serum growth hormone concentrations rose after the hormone was injected A higher level was reached when the hormone was injected intravenously and it remained above the pre injection level for at least 48 hours After the intramuscular injections maximum levels were obtained in 1–4 hours and by 24 hours the concentration had returned to the pre injection levels Additional data is necessary before determination of the half life of growth hormone can be made with certainty

Summary

An antiserum to human growth hormone (Raben) was produced in rabbits and detected by standard immunological techniques The antiserum inhibited human growth hormone both *in vitro* and *in vivo* As tested by single and double agar diffusion methods and using as antigens a crude human pituitary extract the human protein fractions of Raben and human growth hormone no evidence of the presence of more than one antibody in the antiserum was obtained

The antiserum reacted with other preparations of human growth hormone as well as with monkey growth hormone It did not react with growth hormone derived from hog horse or beef There was a minor interaction with fish hormone

The antiserum was used to develop a direct assay of human growth hormones sufficiently sensitive to measure growth hormone in serum Serum growth hormone levels were determined in normal children and adults as well as in patients with acromegaly and hypopituitarism Additionally, serial assays were made of the serum of hypopituitary patients after the intramuscular or intravenous administration of growth hormone

Acknowledgments

Dr Read and Dr Bryan would like to express their gratitude to Dr Maurice Raben not only for preparing most of the human growth hormone as well as the human pituitary protein fractions used in these experiments but also for his continued interest and encouragement

They would like to thank Dr Alfred Wilhelmi for supplying ox, pig horse fish monkey and human growth hormone and Dr Choh Hao Li for his gift of human growth hormone Dr Read thanks Dr Richard Feinberg for a demonstration of the Boyden haemagglutination method as he adapted it for the assay of insulin Dr Read is grateful to Dr Edwin Astwood who at the early planning stage of these experiments in 1956 so generously offered his encouragement and co operation The authors also thank Dr John Opitz who when a medical student carried out the initial immunizations They are especially grateful to Mrs Sandra Little who rendered valuable technical assistance

REFERENCES

AUGUSTIN R and HAYWARD B J (1955) *Int Arch Allergy* 6 154
BOYDEN S V (1951) *J exp Med* 93 107
FINNEY D J (1952) *Statistical Method in Biological Assay* p 21 London Griffin
GREENSPAN F S LI C H SIMPSON M E and EVANS H M (1949) *Endocrinology* 45 455
HAYASHIDA T and LI C H (1958) *Science* 128 1276
OUDIN J (1952) *Meth med Res* 5 335
PREER J R (1956) *J Immunol* 77 54
RABEN M S (1957) *Science* 125 883
RAMON G LEMETAYER E and RICHOU R (1935) *Rev Immunol* 1 199
READ C H (1960) *Progr clin Endocr* in press
READ C H and BRYAN G T (1960) *Recent Progr Hormone Res* 16 in press

DISCUSSION

Beck I should like to compliment Prof Read on a beautiful presentation of a new method in which we have also been interested (Beck J C Fishman J B and McGarry E E (1960) *Proc Soc exp Biol* (*N Y*) in press) We have used a slightly different technique and have succeeded in setting up antibodies to bovine porcine and human growth hormone and also porcine ACTH and bovine TSH In our experience as in Prof Read s there is no cross reactivity between the various types of growth hormone i e bovine porcine or human growth hormone We couple our antigen to red cells with bis-diazotized benzidene rather than tannic acid The method was established in our laboratory and we suspect it increases the sensitivity of the assay We did not kill our rabbits but pre served them in the hope that we can use them for continued production of antiserum We have had no cross reactivity with any other form of protein substance that we have been able to test for in

the assay system Porcine ACTH cross reacts with human ACTH in other words there is hormone specificity but not species specificity Bovine TSH cross reacts with bovine growth hormone and bovine ACTH with bovine gonadotropin However the amount which is required to produce haemagglutination inhibition of these cross reacting substances is 10 µg as compared with only 1 0 µg of the original antigen

Prof Read has opened up a completely new field in endocrine investigation

Read With respect to Dr Beck s comments it is possible that Dr Raben s growth hormone preparation is contaminated with minor amounts of ACTH although we were unable to demonstrate this by adsorption or by single or double gel diffusion techniques There was of course a reaction between growth hormone antibody and ACTH but our data indicated that this was due to contamination of the ACTH with growth hormone rather than *vice versa* Of particular interest in this respect were the adsorption studies to which I alluded but which I did not present When the growth hormone antiserum was adsorbed with ACTH the titre of the antiserum to growth hormone and to ACTH fell Similarly if the antiserum was adsorbed with growth hormone the titre to both hormones was reduced With sufficient numbers of adsorption the antibody level was reduced to zero Dr Beck s results therefore may also reflect the contamination of ACTH with growth hormone

We attempted to produce an antiserum to ACTH but were unsuccessful in doing so because the animals sickened and died

Russell Fraser We have only just begun to attempt this assay and I would like Prof Read to comment on a few practical questions

(i) Have you any experience of preparing the immunized rabbits with other types of growth hormone preparation and are they apparently equally pure with respect to immunizing properties (for instance Prof Wilhelmi s or Prof Li s preparations) ?

(ii) I note that you use guinea pig serum this must be an indifferent neutral serum Might it well be any other animal as long as it was neutral to the two procedures ?

(iii) I should like to make a plea on the question of units It is very nice if possible to stick to the traditional per litre which is common in medicine and here we have been asked to talk of m µg /ml when we might well say µg /l May I ask before that gets too far that it should not be carried on unfortunately it has been the custom with insulin assays to introduce all sorts of confusing terms of this order

(iv) The assays of human sera were of great interest It is of considerable interest that in acromegaly the assay levels come down so

near to the upper limits of normal which makes one wonder whether this does not suggest that the active form in which the hormone circulates may be modified from the form to which your antibody can attach Perhaps a measure of the immunologically active material is not quite the same thing as biological assay I wonder if at this early stage in our study of assays of growth hormone it would not be important to combine the immunological method with the bioassay *Have* you any information on this point, Prof Read from your own patients ?

(v) A point of great interest to many of us who would like to follow in your steps is whether you have any evidence on how this hormone might be concentrated from the serum or perhaps also, from the urine It is customary at the moment to prefer to assay hormones on serum—I have an old fashioned preference for urine on the basis that you can get the 24 hour production more efficiently from the urine than you can from the serum

Read We have not attempted to immunize with other growth hormone preparations Secondly the guinea pig serum is simply a readily available neutral protein and is essential to stabilize the tannic acid growth hormone treated cells Thirdly I think your point with respect to expressing the results is well taken in future we plan to record the values as µg /100 ml

The degree of activity present in an acromegalic is often a difficult clinical assessment The patients with acromegaly whose assays were presented were judged to be active on the basis of their clinical data However we are continuing to investigate this problem in collaboration with a group at the Mayo Clinic We have not done any bioassays by the tibia test method or by any of the other methods which were so eloquently reviewed by Dr Jane Russell in 1944 I believe that Dr Gemzell has carried out tibia test bioassays in patients with acromegaly

Russell Fraser We have found that an insulin tolerance test done *with 0 3 units/kg body weight which is three times the usual* may not be a bad indicator of activity in acromegaly Short of anything better it would be of great interest to see whether your acromegalic patients with rather low values show insulin resistance by this test Our general impression is that acromegaly if it is acromegaly, is active

Gemzell I had the opportunity to work in Prof Li s laboratory in 1957 and was there able to evolve a method of concentrating human growth hormone from human plasma (Gemzell C A (1959) *J clin Endocr* 19 1049) Since then I have been working in Stockholm with this method trying to estimate the normal levels of growth hormone in the human With Dr Luft I have also tried to deter

mine the biological half life of growth hormone in the human The concentration technique for human growth hormone is quite simple It was found by electrophoresis that human growth hormone migrates with albumin in human plasma while bovine growth hormone migrates with globulin The method I use is to treat freshly drawn plasma with the same amount of 10 per cent trichloro acetic acid The precipitate formed is washed three times with 5 per cent trichloroacetic acid The trichloroacetic acid precipitate is then extracted three times with cold absolute ethanol each time the volume of ethanol used is the same as the original plasma volume The pH of the combined ethanol extracts is adjusted to 6 1 by care ful addition of 0 1 N NaOH The precipitate formed which contains albumin and the growth hormone is suspended in cold water di alysed and lyophilized About 200 mg of this plasma fraction is injected into each assay rat for quantitative determination by means of the tibia test The plasma fraction is not toxic to the rats With this method I have been unable in samples of plasma of about 40–50 ml to detect growth hormone in normal individuals In the plasma of acromegalic patients however growth hormone activity was found Our values are about ten times as high as those of Prof Read In one case we have found levels of up to 1 200 µg /100 ml of plasma but usually we found levels of 300–400 µg I have a feeling that the method is good for all levels above 200 µg growth hormone/ 100 ml of plasma

Besides these patients with acromegaly we have found growth hormone activity in the plasma of patients with juvenile type diabetes and also following surgical operation In three cases with gonadal dysgenesis (retarded growth and high levels of total gonado tropins in urine) high plasma levels of growth hormone were found

I have also tried to determine the biological half life of growth hormone Fifty mg of growth hormone was injected intravenously into two normal and four hypophysectomized patients Although the results obtained are not very consistent they agree with those of Prof Read in that the level stays high for quite a long time On com bining all the results we found that the biological half life was about 9 hours which compares favourably with the results obtained by Van Dyke and his co workers who were using parabiotic rats (Van Dyke D C Simpson M E Li C H and Evans H M (1950) *Amer J Physiol* 163 297)

Loraine Prof Read I was very interested in your mathematical treatment of results The short formula that you used to calculate fiducial limits of error may well be a great advantage over the more complicated formulae which have previously been employed I wonder whether in addition to calculating the fiducial limits you

had been able to estimate the index of precision lambda Also was it possible to test the accuracy of the method by conducting experiments in which the growth hormone was added to urine and the percentage recovered calculated?

Read Finney (1952 *loc cit*) described four types of assay The simplest type is the direct assay in which, as in our system the end point is an all or none response in a single individual A second type of all or none assay is the more familiar quantal assay Then there are the graded response assays which are either parallel line or slope ratio assays

In direct assays it is not possible to derive a lambda We have carried out only one recovery experiment so far although we plan to do more In this instance there was an almost 100 per cent recovery

Wilhelmi Prof Read you showed us in Fig 14 the injection of 4 mg growth hormone did you calculate the volume of distribution?

Read No

GROWTH HORMONE AND THE MOBILIZATION OF FATTY ACIDS

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The association of the action of growth hormone with increased mobilization of fat is well supported by many studies over the past quarter century (Lee and Schaffer 1934 Best and Campbell 1936 Levin and Farber 1952 Greenbaum and McLean 1953) Our interests have been concerned with the details of the process by which the hormone releases fat, its inhibition by glucose and insulin and with the problem of whether this effect is primarily related to the anabolic action of growth hormone or is merely a consequence of it The findings to be presented here contain new information related to these subjects and suggest possible directions of further studies

Growth hormone and unesterified fatty acids of plasma

The emergence of a concept of fat mobilization which emphasized the major contribution of unesterified fatty acids in the transfer of lipid for energy requirements (Dole 1956 Gordon and Cherkes 1956 Gordon 1957) permitted a closer inspection of the influence of growth hormone It was soon found that growth hormone increased the unesterified fatty acids in the plasma (Raben and Hollenberg 1958 1959*a* Raben 1959) and this effect has also been seen by others (Engel *et al* 1958 Knobil and Greep 1959 Wilgram *et al* 1959) In both dog and man the high concentrations during fasting were readily increased two or three fold with growth hormone but glucose or food suppressed the effect even when

large amounts of hormone were given In the alloxan diabetic dog, however food was much less effective in preventing the effect of the hormone These findings and the fact that small amounts of growth hormone were effective made it likely that growth hormone was important in the physiological control of the caloric supply provided by adipose tissue in the

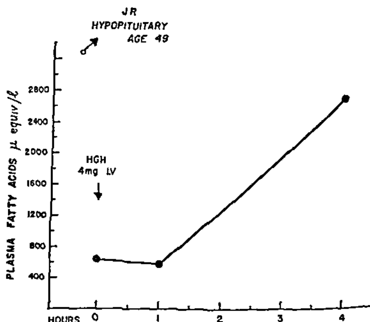


FIG 1 Time of response of plasma fatty acids after intravenous human growth hormone in fasted hypopituitary subject

form of unesterified fatty acids Growth hormone however is not essential for the release of fatty acids for an increase in the blood value occurred with fasting in hypopituitary subjects but in both these subjects and in normal individuals the hormone accelerated the release of fatty acids beyond the rate induced by *fasting alone*

In the dog plasma fatty acids were increased by porcine bovine human and simian growth hormone but in man only human and simian preparations were effective The action

was rapid but not immediate Intravenous administration of 4 mg of acetic acid extracted human growth hormone (Raben 1957) to a hypopituitary patient (probably at least 10 times the minimal effective dose) produced no rise of fatty acids in 1 hour but more than a fourfold increase in 4 hours (Fig 1)

Further evidence that increased mobilization of fatty acids occurs with growth hormone

With the knowledge that growth hormone tends to promote ketosis lower respiratory quotient diminish fat stores and cause a rapid transfer of fat from adipose tissue to the liver, the increase of fatty acids in the plasma was assumed to result from augmented output by adipose tissue This assumption is now supported by several experimental observations Brown and Bennett (1959) measured the conversion of ^{14}C labelled palmitic acid to ^{14}CO in human subjects with and without human growth hormone The percentage of the label appearing as ^{14}CO was not changed by the hormone, but as there was a greater quantity of plasma fatty acid after the hormone, the total conversion of plasma fatty acid to CO_2 was greater after growth hormone It was also noted that glucose was less effective after growth hormone in decreasing the conversion of ^{14}C palmitate to ^{14}CO Knobil (1959) incubated adipose tissue removed from hypophysectomized rats and found an increased output of unesterified fatty acids *in vitro* with fat from animals receiving growth hormone, as judged by the total amount of unesterified fatty acid present in tissue and medium at the end of incubation

Experiments were performed confirming the results of Knobil (Fig 2) In addition it was seen that the behaviour of epididymal fat was affected by pretreatment of the rat with very small doses of hormone and by comparing the titratable fatty acid present in the fat at the time of removal from the animal with the amount in the medium and tissue after incubation an estimate of net production or loss of fatty acid during the interval of incubation could be made (Fig 3)

The tissue was removed from live rats anaesthetized with

sodium pentobarbital after 24 hours without food and 18 hours after the last injection of growth hormone During a 3 hour incubation of tissue from untreated hypophysectomized rats, there was little change or a loss of unesterified fatty acid, indicating that the combined processes of esterification and metabolism of acid equalled or exceeded the rate of lipolysis Since the tissue must have been releasing fatty acids at

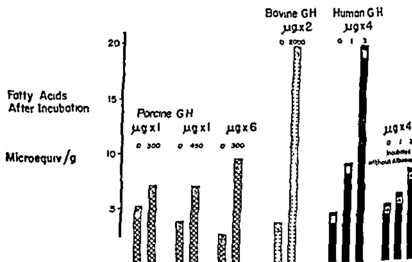


FIG 2 *Effect of growth hormone administered to hypophysectomized rats on fatty acid output of excised adipose tissue* Daily dose and number of injections are indicated Rats were fasted 24 hours and last injection of growth hormone was 18 hours before rats were anaesthetized with sodium pentobarbital fat removed and incubated 3 hours at 37 in Krebs Ringer phosphate 5 per cent albumin Albumin was omitted as noted in group to far right Columns indicate the average total unesterified fatty acid of medium and tissue at the end of incubation for the number of trials specified.

the time of removal from the fasting animals it would seem that either the *in vitro* environment was inadequate for continuation of this process or an influence of transient character was operative in the intact animal Circulating adrenaline or sympathetic nerve activity in the animal might explain the difference, since adrenaline is a very potent stimulator of fatty acid release During incubation of epididymal fat from

hypophysectomized rats which had received growth hormone a net production of fatty acid occurred (Fig 3) The effect was seen after four daily injections of as little as 1 0 μg of human growth hormone The concentration of fatty acid at the time of excision of the tissue was not affected by pre treatment with these small doses (Fig 3)

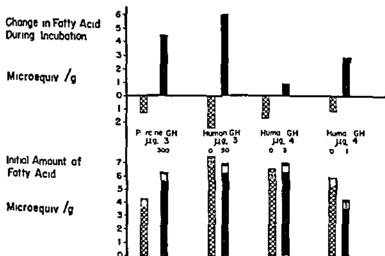


FIG 3 Fatty acid output during incubation of adipose tissue from treated and untreated hypophysectomized rats One piece of tissue from each animal was extracted and titrated immediately (initial amount) and another piece was titrated tog ther with medium at the end of 3 hour s incubation The change is the final minus the initial value The value for the change would be somewhat more positive than indicated if the final concentration were calculated from the weights of tissue before rather than after incubation.

The adipose tissue was shaken in a water bath at 37 in Krebs Ringer phosphate buffer containing 5 per cent human albumin pretreated to remove fatty acids the gas phase was air Long chain fatty acids have a strong affinity for albumin and are transported in the blood bound to this protein Albumin in the medium promotes the transfer of fatty acids from within the adipose tissue to the medium and it is desirable

to provide this acceptor of fatty acids when production of fatty acid is being measured, and particularly if large amounts are being formed, since the accumulation of high concentrations within the fat may slow the rate of further production

It seemed possible that when, as in the experiments cited a minimal stimulation of fatty acid production was compared with control specimens in which acids were either not changing or decreasing within the tissue during incubation it might be better to omit albumin from the medium, since a sizeable fraction of the fatty acids initially present in the adipose tissue was rapidly transferred to albumin The firmly bound fatty acids might then not be available to the tissue for metabolism and esterification and the maximal reduction in fatty acids would thus be limited The rapid transfer of fatty acid from tissue to medium was seen when fat from three fasted hypophysectomized rats was incubated in medium containing albumin freed of fatty acids After 20 minutes the fatty acid content of the adipose tissue averaged 3 1 microequiv /g of tissue and there were 2 7 microequiv /g in the medium After 3 hours the acid in the tissue fell to 1 1 microequiv /g while no further change occurred in the medium

As may be seen in Fig 2 the logic for omitting albumin was not supported by the results of a small number of experiments designed to test this point perhaps because the absence of albumin restricted the production of acid in tissues from treated rats more than it enhanced the disappearance of acid in untreated tissues In a few observations on tissue from normal rats fasted for 16–24 hours it was seen that production and loss of fatty acid during incubation occurred with about equal frequency in samples from different rats and when a loss of acid occurred it was exaggerated by the absence of albumin in the medium but when there was production of acid it was augmented by the presence of albumin

In the experiments in which initial and final values of fatty acid were determined the final concentrations were calculated for the weight of fat after incubation Since an increase in weight of the tissue occurs during incubation the final

values may have been about 20 per cent too low, but the conclusions would not be changed by correcting for this dif ference

Effect of growth hormone added IN VITRO on fatty acid mobilization

In most of our studies with isolated adipose tissue, the titratable acid was measured only at the end of the incubation period and in the early experiments we assumed as have others in similar studies that the amount found was the quantity produced during the incubation Fortunately this misconception does not invalidate interpretations about the effect of added agents since treated and control tissues were from the same animal and a higher final value still indicated a change towards greater output of fatty acid

After incubation of tissue from normal rats for 3 hours in Krebs Ringer phosphate buffer—5 per cent albumin and titrating tissue and medium together there was more fatty acid found with fasted than fed tissue but when the change during incubation was determined it was seen that the final values did not correlate with production of acid The tissue from fed animals had less acid at the time of excision than tissue from fasted animals and the amount sometimes in creased and sometimes fell during incubation of both types of tissue The addition of growth hormone preparations to the medium increased the amount of acid found after incubation and fasting the rat for 18–24 hours increased the sensitivity of the adipose tissue to this effect In Table I the response to acetic acid extracted human growth hormone (GH) is shown The minimal effective amount of hormone was ap proximately 10 µg in the 1 ml of albumin containing medium Bovine (NIH BGH 1) and porcine (acetic acid extracted) prep arations of growth hormone were active in similar doses

The amount of growth hormone required for an effect *in vitro* while considerably less than that reported by White and Engel (1958) was proportionately more than was needed for fatty acid mobilization when given *in vivo* While there

has been no reason to doubt that the effect of preparations administered *in vivo* on plasma fatty acids and on the fatty acid output of excised adipose tissue was due to the growth hormone itself, this is not equally true for experiments in which growth hormone was added *in vitro* Studies with pituitary preparations have been plagued by the extreme sensitivity

Table I

EFFECT OF GROWTH HORMONE PREPARATIONS ADDED TO MEDIUM ON FATTY ACID PRODUCTION BY RAT EPIDIDYMAL FAT INCUBATED FOR 3 HOURS IN 1 ML. KREBS RINGER PHOSPHATE 5 PER CENT ALBUMIN IN EACH CASE THE EFFECT OF THE HORMONE WAS COMPARED AGAINST UNTREATED TISSUES FROM THE SAME ANIMALS TISSUE AND MEDIUM WERE TITRATED TOGETHER

	Growth hormone in medium (μg)	*No of determinations*	*Fatty acid after incubation* (microequiv /g)
Human GH	0	10	10
	30	10	18 5
	0	6	7 7
	100	6	27 3
NIH bovine GH 1	0	4	7 7
	10	4	8 1
	0	2	5 ~
	30	2	12
	0	6	12
	500	6	36
Bovine GH (Armour R285–128)	0	10	7 8
	5	10	19 8

of rat adipose tissue to the direct addition of corticotropin which, from Astwood s (1958) data seems likely to be active in amounts of less than 0 01 μg The acceptance of the *in vitro* effect of growth hormone has been further complicated by evidence of differences in the potency of preparations with similar growth promoting activity For example an older bovine preparation (Armour R285–128) seemed more potent than the recent NIH BGH 1 (Table I)

Isolated human adipose tissue was used in the hope that it would distinguish human growth hormone from the growth

hormone of other species and thus help decide whether the *in vitro* activity was due to growth hormone itself Human subcutaneous fat and when tested on one occasion omental fat, were sluggish compared to rat tissue Adrenaline was regularly active in promoting fatty acid output, but the amounts produced were very much smaller than those produced by adipose tissue from the rat We had found that β mercaptoethylamine was active in stimulating fatty acid release from rat

Table II

Fatty acid production of human subcutaneous fat Tissue was removed during operative procedure and treated like rat tissue in Table I

	Amount added to medium (µg)	*No of determinations*	*Fatty acid after incubation* (microequiv /g)
Human GH	0	10	2 1
	100–1000	21	2 5
Bovine GH (Armour R285–108)	0	12	2 0
	100–1000	12	2 1
Human and porcine corticotropin	0	16	1 8
	100–1000	18	2 0
Adrenaline	0	12	1 7
	100	12	4 3

adipose tissue (Raben and Hollenberg 1959*b*) and this was also effective with human tissue However neither corticotropin nor growth hormone of any origin produced a definite effect on human tissue (Table II) The ineffectiveness of these substances was not attributable to the area from which the fat was taken since both subcutaneous and retrorenal fat of the rat were found responsive to these preparations

Mechanism by which glucose and insulin decrease output of fatty acids

An attempt to understand the action of growth hormone in favouring the release of fatty acids into the circulation appears inseparable from an appreciation of the way it is opposed by

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	10	4	8 1
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	30	2	12
	0	6	12
	500	6	36
Bovine GH	0	10	7 8
(Armour R285–128)	5	10	19 8

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Isolated human adipose tissue was used in the hope that it would distinguish human growth hormone from the growth

of tissue acid there was usually also increased loss of acid from the medium These changes would be expected if esterification was promoted by the glucose and insulin Glucose alone in the higher concentrations (Fig 4) had a similar effect while insulin alone did not With 100 mg per cent of glucose in the medium progressively greater changes occurred with

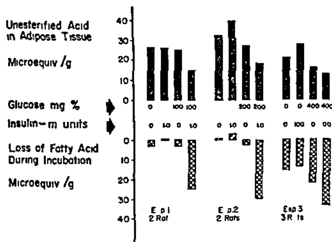


FIG 4 Effect of glucose and insulin on the uptake and esterification of added oleic acid by rat epididymal fat Four pieces of fat from each fed normal rat were compared with different supplements to medium Tissue was incubated 3 hours at 37 in Krebs Ringer phosphate 5 per cent albumin containing 10 microequiv oleic acid in one ml Tissue and medium were titrated separately at the end of incubation The upper bars indicate the concentration of unesterified fatty acid in the tissue and the lower bars the amount of titratable fatty acid disappearing during incubation

increasing amounts of insulin (Fig 5) and an effect was usually detectable with as little as 30 microunits

The evidence that enhanced metabolism of glucose by adipose tissue inhibits the release of fatty acids by promoting esterification rather than by depressing lipolysis of triglycerides correlates well with the findings that the lipoprotein lipase activity is greater in the adipose tissue of fed than of

glucose and insulin The infusion of glucose in fasted normal dogs and glucose and insulin in diabetic animals precipitously lowers the plasma fatty acids despite pretreatment with large amounts of growth hormone Our studies suggest that the abundant availability of carbohydrate to adipose tissue restricts the release of unesterified fatty acids by promoting esterification of free acid

It was shown by Gordon and Cherkes (1958) that glucose and insulin diminished the fatty acid output of isolated adipose tissue as judged by the amount of acid in the medium at the end of incubation The pattern of the response becomes clearer however when the total amount of acid present at the start and end of incubation is known During incubation of fat from 16 to 24 hour fasted normal rats in Krebs Ringer phosphate 5 per cent albumin both increases and decreases of fatty acid were seen With glucose and insulin present, there was regularly a decrease of fatty acid and in the case of tissues which lost acid even in unenriched medium, a greater fall occurred with glucose and insulin Since the addition of glucose and insulin would not have encouraged the metabolism of fatty acids it was assumed that the missing fatty acids were esterified This view was further supported by experiments in which the changes were exaggerated by the addition of high concentrations of fatty acid to the medium

Epididymal fat from fed and 18 hour fasted rats cut into pieces weighing about 80–100 mg was incubated in 1 0 ml Krebs Ringer phosphate 5 *per cent* albumin containing 10 microequiv of oleic acid After 2 or 3 hours there was loss of acid from the medium usually with more than half the loss accounted for as free acid in the fat The concentration of acid in the adipose tissue was three or four times that in the medium and the effect of glucose and insulin was to lower the concentration of free acid in the tissue and to cause a greater total disappearance of acid (Figs 4 and 5) With the smaller doses of glucose and insulin the decreased concentration of acid in the fat was not necessarily accompanied by a greater loss from the medium but with larger amounts and a greater decrease

Relation of the release of fatty acids to the anabolic effect of growth hormone

Both forced feeding and administration of insulin have been reported to produce nitrogen retention and growth in hypophysectomized rats but only with sufficient food to cause excessive formation of fat Thus an ample caloric supply to tissues may of itself be anabolic Growth hormone induces anabolism and growth without the caloric wastage of fat formation and it would be of interest to know whether the provision of abundant calories in the form of fatty acids is an important part of its anabolic action There is no adequate evidence to decide whether the mobilization of fatty acids is a primary action of growth hormone or whether it is a consequence of another mechanism of anabolic effect The extreme sensitivity of the fatty acid response to growth hormone suggests its importance In our earlier studies an effect on plasma fatty acids was seen in a hypopituitary subject with less than 10 µg of human growth hormone per Kg and in the present work 1 µg per day affected the fatty acid output of adipose tissue removed from hypophysectomized rats These are probably the smallest doses of growth hormone shown to have a biological effect

The mechanism by which growth hormone augments the release of fatty acids from adipose tissue remains obscure Evidence is presented that glucose and insulin limit the release by favouring esterification of fatty acids and it may be of interest to study the action of growth hormone on this process In any case detailed information about the mechanism of action of growth hormone promises to become increasingly accessible and a more knowledgable discussion should soon be possible

REFERENCES

ASTWOOD E B (1958) Personal communication
BEST C H and CAMPBELL J (1936) *J Physiol (Lond)* 86 190
BROWN J and BENNETT L R (1959) *J clin Invest* 38 993
DOLE V P (1956) *J clin Invest* 35 150

fasted rats, and that glucose and insulin added *in vitro* prevent the fall of lipolytic activity during incubation which otherwise occurs (Hollenberg 1959*a* and *b*) The data are also consistent with the work of Shapiro Chowers and Rose (1957) indicating that adipose tissue from fed rats esterifies fatty acids more readily than tissue from fasted rats Addition

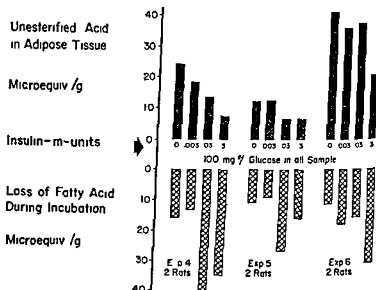


FIG 5 Effect of increasing amounts of insulin in the presence of glucose on concentration of unesterified fatty acid in adipose tissue and on disappearance of added oleic acid Tissues were incubated 2 hours but other conditions were the same as for Fig 4 Fed normal rats were used in Experiment 5 and 18 hour fasted animals in Experiments 4 and 6

of glucose to incubating slices of rat mammary gland was shown by Hirsch Lossow and Chaikoff (1956) to increase esterification of fatty acids and thus the enhancement of esterification by carbohydrate metabolism may be a more general phenomenon among tissues acting to minimize the metabolism of fat both by restricting the output of fatty acids from adipose tissue and by promoting storage rather than use of the available acids in other tissues

Russell Fraser Does your difficulty in obtaining any *in vitro* release of unesterified fatty acids from the epididymal fat pad with growth hormone suggest that if you added serum from an animal injected with growth hormone you might obtain a different result?

Raben That has not been tested adequately The doubt about the *in vitro* effect of growth hormone on release of fatty acids arises from the large dose required and from the difference of effectiveness of various growth hormone preparations

When fat is taken from a fasted rat it is taken at a time when the fat must be providing large amounts of fatty acids yet it produces only a small amount during incubation so that the *in vitro* behaviour is not simulating the behaviour at the time of removal Some factor which is operative in the animal and which is transient may be missing or else the *in vitro* environment is not satisfactory One possible difference worth considering is the absence of sympathetic nervous activity when the fat is excised

Young Dr Raben do you consider that there may be an increased secretion of growth hormone during fasting?

Raben There would not have to be an increase to control fatty acid output because if the growth hormone were constant the meal would suppress any fat mobilizing effect it had and it could be operative between meals to mobilize extra calories This provides a mechanism in which growth hormone would not have to fluctuate and yet would be able to control the rate of mobilization of fatty acid between meals

Young What about longer fasting?

Raben I cannot say

Beck Dr Raben is there any information from work with the epididymal fat pad as to whether the age of the rat makes any difference to the growth hormone response to the *in vitro* addition of growth hormone?

Raben Since I am not certain about the *in vitro* effect of growth hormone at any age I cannot say but older rats may have less active fat and be less responsive

Beck It is remarkable that huge quantities of growth hormone are required *in vitro* when about 0 5 mg in a 70 kg hypopituitary patient led to such a marked increase I wonder whether everyone has been using epididymal fat pads from older animals without doing it in younger animals

Raben Most of our experiments were with 150 to 200 g rats

Wilhelmi With respect to Dr Beck's question although this does not concern growth hormone Dr Ball and his co workers have published data showing that as far as the insulin effect on the epididymal fat pad is concerned age is very important (Hagen J M

ENGEL H R HALLMAN L SIEGEL S and BERGENSTAL D (1958) *Proc Soc exp Biol (N Y)* 98 753
GORDON R S Jr (1957) *J clin Invest* 36 810
GORDON R S Jr and CHERKES A (1956) *J clin Invest* 35 206
GORDON R S Jr and CHERKES A (1958) *Proc Soc exp Biol (N Y)* 97 150
GREENBAUM A L and McLEAN P (1953) *Biochem J* 54 507
HIRSCH P F LOSSOW W J and CHAIKOFF I L (1956) *J biol Chem* 221 509
HOLLENBERG C H (1959*a*) *Fed Proc* 18 274
HOLLENBERG C H (1959*b*) *Amer J Physiol* 197 667
KNOBIL E (1959) *Proc Soc exp Biol (N Y)* 101 288
KNOBIL E and GREEP R O (1959) *Recent Progr Hormone Res* 15 1
LEE M O and SCHAFFER N K (1934) *J Nutr* 7 337
LEVIN L and FARBER R K (1952) *Recent Progr Hormone Res* 7 399
RABEN M S (1957) *Science* 125 883
RABEN M S (1959) *Recent Progr Hormone Res* 15 71
RABEN M S and HOLLENBERG C H (1958) *J clin Invest* 37 9??
RABEN M S and HOLLENBERG C H (1959*a*) *J clin Invest* 38 484
RABEN M S and HOLLENBERG C H (1959*b*) *J clin Invest* 38 1032
SHAPIRO B CHOWERS I and ROSE G (1957) *Biochim biophys Acta* 23 115
WHITE J E and ENGEL F L (1958) *J clin Invest* 37 1556
WILGRAM G F CAMPBELL J LEWIS L and PATTERSON J (1959) *Diabetes* 8 205

DISCUSSION

Li Dr Raben do you say that ACTH has no *in vivo* effect ?

Raben ACTH has little if any effect on plasma fatty acids when injected in man The value was a little higher than in the controls

Li Your previous studies indicated that ACTH is about 1 000 times more active than growth hormone in mobilizing fat from depot to liver in the mouse but now have you found that the effects of both in the human are about the same in this respect ?

Raben The plasma values of unesterified fatty acids increased in man 2–4 hours after intramuscular injection of 0 5–4 0 mg of human growth hormone but were little affected by an equal amount of ACTH

Li I recall that at one time you interpreted the adipokinetic effect of growth hormone as a contaminating factor In view of this current observation do you think that your proposed contaminating factor responsible for the fat mobilizing effect in ACTH and in growth hormone is a different one in each of the two hormones ?

Raben The factor which when injected increases unesterified fatty acids seems to be growth hormone itself

physectomized diabetic animals to see what lack of insulin would do to the system?

Raben Drs R O Scow and E B Astwood studied adipose tissue from totally depancreatectomized rats There was a large amount of free acid in the tissue at the time of excision (about 25 μequiv /g as I recall) yet the output during the incubation period was small It was rather like an accentuation of what occurs in fasting animals

Beck We also gave growth hormone to acromegalics and we observed a very slight rise it is very much less dramatic than when given to the normal fasting individual Incidentally obese individuals are very resistant to the injection of growth hormone during fasting The plasma unesterified fatty acid is high to start with and one can increase it but the slope of the increase is very much less than in normals

Wilhelmi Dr Raben have you made any observations on the effect of growth hormone on unesterified fatty acids in normal children?

Raben No

Wilhelmi Dr D Bergenstal told me that he tried human growth hormone in normal children and got no response—as if they were already well saturated He was looking I believe for nitrogen retention

Russell Fraser We have seen a rise in unesterified fatty acids in a ten year old boy who was a normal subject in this respect

Dr Raben have you any information on whether the extent of the response is in any way influenced by the basal level? One might expect that it would not be

Raben I do not know

de la Balze If you give such a patient or an animal growth hormone and then add thyroid hormone do you then get an increase of unesterified fatty acids?

Raben That was not done It is rather remarkable that the response to growth hormone is so dependable considering the large number of factors that affect the level of unesterified fatty acids including adrenaline fever hyperthyroidism and circumstances demanding more calories Fatty acids in normal individuals after an overnight fast change very little during a 4 hour period but almost invariably rise with growth hormone Conditions that lead to increased plasma fatty acids are often those in which more calories are required by the tissues but perhaps with growth hormone more calories are provided than are being demanded

Ball E G and Cooper, O (1959) *J biol Chem* 234 781) In the older and heavier rats this effect is reduced to quite a small proportion of the optimal effect which occurs in rats of about the order of 150–180 g Perhaps the other responses might be expected to be similarly reduced

Russell Fraser It is equally true that stimulus of the sulphate uptake by cartilage is very strikingly related to age

Read We have used the epididymal fat pad to study the effect of human growth hormone on disappearance of glucose (not on fat) and the production of CO_2 and have been unable to find any effect of it whatsoever in concentrations of 25 m μg /ml up to 1 μg /ml

Raben Winegrad Shaw and Renold found an increase in glucose uptake by adipose tissue and increased conversion to CO_2 with 0 2–1 0 mg growth hormone/ml (Winegrad A I Shaw W N and Renold A E (1958) *J clin Invest* 37 943)

Luft Dr Raben were you able to measure any release of triglycerides into the medium in the *in vitro* system when you used fasting animals ?

Raben I have not measured that but one would have to be sure it is not from the cut edges

Beck We have some information about the extreme delicacy of this inhibition of the growth hormone induced rise in unesterified fatty acid with glucose For instance in the normal individual if you give 5 mg growth hormone after a suitable period of fasting and a constant infusion of glucose e g 3 g /hr there is the expected rise which one saw when no glucose was given If one then repeats this experiment with a 6 g /hr glucose infusion the growth hormone no longer has an effect on the unesterified fatty acids in the same dose therefore it must be a very delicate balance

de la Balze Dr Raben have you carried out electrophoresis of the animal serum and Sudan black staining ?

Raben We have not studied it

Malinow Have lipids other than unesterified fatty acids been measured e g cholesterol ?

Raben Wilgram reported that only unesterified fatty acids were rapidly affected but after several days of growth hormone injections in dogs other blood lipid components rose (Wilgram *et al* 1959 *loc cit*)

de la Balze Have you tried this with the serum of acromegalic patients ?

Raben I have injected growth hormone twice into acromegalic patients and have not obtained a rise in plasma unesterified fatty acids

Pearson Have you studied the isolated fat in diabetic and hypo

Once preparations of growth hormone active in man i e human growth hormone became available it was possible for the first time to study its effect on metabolic processes in human subjects

We have been interested in this problem and performed some studies with human growth hormone in different groups of patients The growth hormone preparation used by us was prepared by Gemzell according to the procedure of Li and Papkoff Biological testing in hypophysectomized rats showed that human growth hormone and bovine growth hormone are equally active on a weight basis Furthermore human growth hormone contains only insignificant amounts of adrenocorticotropic hormone (ACTH) (less than 30 μg /mg) and thyroid stimulating hormone (TSH) (0 5 U S P units/ mg) As expected these amounts were of no significance in clinical work Ten milligrams given intramuscularly daily for 12–16 days did not influence the adrenocortical activity or thyroid function of four patients studied in these respects (Table I) Nor could any measurable amounts of oxytocin or vasopressin be demonstrated in our preparations (tested at levels of 10 and 5 μg /mg respectively)

The results obtained with this growth hormone preparation are presented below

Protein metabolism

Ten milligrams of human growth hormone were given daily for 12–16 days to four adult subjects on a complete metabolic study (Ikkos Luft and Gemzell 1958 1959*a*) This was followed by a prompt anabolic action as shown by a retention of nitrogen phosphorus and potassium This retention persisted during the whole period of growth administration Upon withdrawal of growth hormone the retention of nitrogen phosphorus and potassium stopped and the balances became negative It is of interest however that the loss of nitrogen during the post treatment period was significantly smaller than the retention during growth hormone administration

ASPECTS OF THE METABOLIC ACTION OF HUMAN GROWTH HORMONE*

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OUR knowledge of the effect of growth hormone (GH) in man has hitherto almost exclusively been indirect, and based on observations in patients with acromegaly and pituitary insufficiency Growth hormone prepared from animal pituitaries has sometimes caused metabolic alterations in man similar to those observed in animals but has usually been inactive (see Smith, Gaebler and Long, 1955, Astwood 1955) Thus the situation with respect to growth hormone differed from that in other pituitary hormones which prepared from animal glands are generally active in man as well

It was suggested in 1955 that the discrepancy between growth hormone and other pituitary hormones as regards their activity in man might be due to a species specificity of growth hormone (Wilhelmi 1955) Data collected since then support this view Human growth hormone has been shown to be metabolically active in man The species specificity of the hormone is confined not only to man but also to some other species In general it can be stated that growth hormone prepared from pituitaries of lower animals is inactive in the higher species whereas growth hormone from higher species is active in the lower animals as well Physicochemical studies have shown differences between growth hormones of different origin which might form the basis of the species specificity (Li and Papkoff 1956)

* This work was made possible by grants from the Swedish Medical Research Council the Swedish Diabetics Association and Knut and Alice Wallenberg s Foundation

Table II

CUMULATIVE BALANCES OF NITROGEN PHOSPHORUS AND POTASSIUM

Case	Period	Duration in days	Nitrogen (g) urine	Nitrogen (g) faeces	Nitrogen (g) Total	Phosphorus (mg) urine	Phosphorus (mg) faeces	Phosphorus (mg) Total	Potassium (m equiv) urine	Potassium (m equiv) faeces	Potassium (m equiv) Total
1	growth hormone	12	+ 38 48	+ 1 80	+ 40 28	+ 1 116	+ 1 500	+ 2 610	+ 295	+ 7	+ 302
	post treatment	16	− 12 54	+ 4 82	− 7 72	− 2 656	+ 544	− 2 112	− 2	+ 5	+ 3
	Total				+ 32 56			+ 504			+ 305
2	growth hormone	16	+ 40 [illegible]	− 0 96	+ 40 32	+ 1 824	+ 208	+ 2 032	+ 190	− 5	+ 185
	post treatment	12	− 1 15	+ 0 92	− 0 23	− 516	− 420	− 936	− 95	− 8	− 103
	Total				+ 40 09			+ 1 096			+ 82
3	growth hormone	14	+ 20 58	− 4 27	+ 16 31	+ 385	− 343	+ 42	+ 308	+ 35	+ 343
4	growth hormone	14	+ 54 53	− 4 20	+ 50 33	+ 1 491	− 266	+ 1 225	+ 175	− 21	+ 154

Table I

ADRENOCORTICAL AND THYROID FUNCTIONS BEFORE AND DURING GROWTH HORMONE ADMINISTRATION

Case	17 Ketosteroids (mg /24 hrs *)		17 Ketogenic steroids (mg /24 hrs)*		Protein bound iodine (µg /)		Initial ^{131}I uptake (H value†)	
	control	growth hormone	control	growth hormone	control	growth hormone	control	growth hormone
1	14 8 (11)	8 2 (12)	15 6 (11)	15 2 (12)				
2	9 2 (12)	9 1 (16)	13 9 (12)	13 6 (16)				
3	7 9 (14)	7 8 (14)	12 1 (14)	10 9 (14)	5 6 7 0	5 0 5 4	+2 6	+2 4
4	3 2 (14)	4 5 (14)	14 8 (14)	19 8 (14)	4 3 4 9	3 4 3 7	−1 4	−0 3

Mean values figures in parentheses denote number of determinations
† Calculated according to Einhorn (19 8)

excess potassium might have been used for new formation and storage of glycogen

Another expression of the anabolic effect of the human growth hormone was the change in creatine excretion (Fig 1) Case 1 had increased creatinuria during the control period

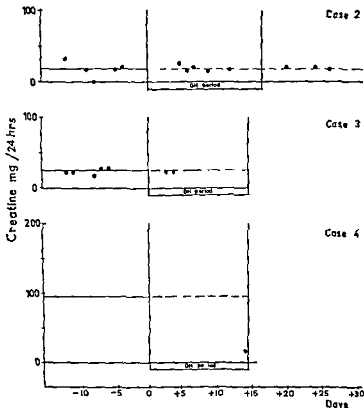


Fig 1b Creatine excretion in urine (mg /24 hours)

most probably because of the patient s rheumatoid arthritis The creatinuria showed a clear decrease during the growth hormone period A smaller but significant decrease in creatine excretion was also observed in Case 4 in which the creatine

The amounts of nitrogen phosphorus and potassium retained during the growth hormone period are shown in Table II The nitrogen retained corresponded to 0 5 to 1 6

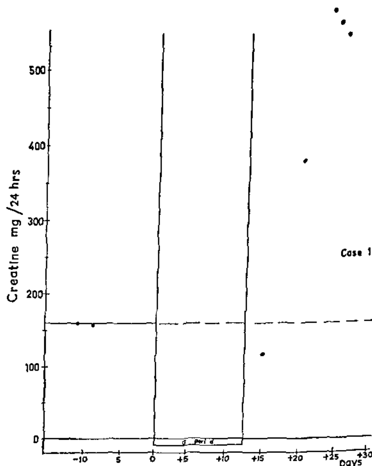


FIG 1a Creatine excretion in urine (mg /24 hours)

kg of fat free protoplasm Calculations showed that the amount of phosphorus retained agreed well with the amount expected from the balances of other elements (nitrogen and calcium) whereas potassium was retained in excess The

The results as regards protein metabolism obtained by different groups are summarized in Table IV The results of the British group (Medical Research Council 1959) are identical with ours The absence of an unequivocal nitrogen retention in one of their cases could be explained by the short duration of growth hormone administration (total dose 15 mg in two days) The difference between the results of Beck and co workers (1958) and those of the two other groups cannot be explained

Calcium balance

Growth hormone significantly influenced the calcium metabolism (Table V) All four patients showed an increased urinary excretion of calcium the loss varying between 650

Table V

CUMULATIVE BALANCES OF CALCIUM

Case	Period	Duration in days	Calcium (mg) urine	Calcium (mg) faeces	Calcium (mg) Total
1	growth hormone	12	888	+ 1 572	+ 684
	post treatment	16	− 496	+720	+ 224
				Total	+ 908
2	growth hormone	16	− 720	− 1 216	− 1 936
	post-treatment	12	− 180	− 1 344	− 1 524
				Total	− 3 460
3	growth hormone	14	− 651	− 714	− 1 365
4	growth hormone	14	− 1 379	− 1 533	− 2 912

and 1 380 mg for a treatment period of 12–16 days The faecal excretion of calcium increased in three of the patients whereas it decreased in Case 1 Consequently the total calcium balance was negative in three cases but positive in one Upon withdrawal of growth hormone the urinary loss of calcium decreased Here as well a discrepancy existed

excretion values were somewhat raised before growth hormone The normal creatinuria of Cases 2 and 3 remained practically unchanged

A further sign of the anabolic action of growth hormone was the decrease in non protein nitrogen (N P N) observed during growth hormone treatment (Table III)

Table III

NON PROTEIN NITROGEN IN BLOOD (MG /100 ML)

Period	*Case* 1	*Case* 2	*Case* 3	*Case* 4
control	40 38 38 38	36 34 33 40	45 42 38	40 43 31
growth hormone	24 30	24 28	22 30 32 27	23 31 27 30

Table IV

EFFECT OF HUMAN GROWTH HORMONE ON PROTEIN AND CALCIUM METABOLISM IN MAN OBTAINED BY DIFFERENT RESEARCH GROUPS

	*Change**	*British group*† (1959)	*Beck* et al (1958)	*Present authors*
nitrogen balance	+	7	3	4
	±	1	1	
	0		2	
phosphorus balance	+		1	4
	±		3	
	0		2	
potassium balance	+	4	2	4
	±		[illegible]	
	0		2	
N P N or urea in blood	decreased	4	1	4
	unchanged			
urinary calcium	decreased			
	increased	8		4
	unchanged			
calcium balance	+		1	1
	±		3	
	0		2	
	−			3

(+) signifies po itive balance (±) a smaller and less significant chang (0) no re ponse (−) n gativ balan e

† Urine studies only

When growth hormone was discontinued these elements were once more lost and the volume of the extracellular fluid decreased

Body weight increased in all patients during the growth hormone period but the weight gained was rapidly lost when growth hormone was discontinued (Fig 2) Since nitrogen was not lost in the post treatment period it is highly probable that the rapid weight changes were due chiefly to changes in the volume of the extracellular fluid and not to changes in the cell mass

The retention of sodium and chloride raised the question whether growth hormone increased aldosterone secretion

Table VIII

ALDOSTERONE IN URINE

Case	Aldosterone in urine (μg /24 hrs) control period	growth hormone period	post treatment period
1	3 0	0 5–3 0–3 0–0 5–4 0–5 0	0 5–0 5
2	1 5	1 5–1 0–1 0–1 0–1 0	2 0–2 0–1 0–1 0
3	5 0	2 0–4 0–2 0	
4	18 0	[illegible] 0–3 0–2 0	

Measurements of urinary aldosterone in the four cases (Table VIII) did not show any changes during the growth hormone period It thus looked as if the observed retention of electrolytes and water was not mediated by aldosterone On the other hand an increased aldosterone excretion has been reported following growth hormone this increase depending in part on the dose of growth hormone (Beck *et al* 1958) We have lately re examined this question in short term studies with 30 mg of growth hormone daily for three days in healthy volunteers With this dose a definite increase in urinary aldosterone was observed (Hokfelt Ikkos and Luft 1959)

between the results of Beck and co workers (1958) and the present authors (Table IV)

Sodium and chloride metabolism

During the growth hormone period there was a significant retention of sodium and chloride and a concomitant increase in the volume of the extracellular fluid The retention of sodium and chloride and the increase in the extracellular fluid were observed in all four cases (Tables VI and VII)

Table VI

CUMULATIVE BALANCES OF SODIUM AND CHLORIDE

Case	*Period*	*Duration in days*	*Sodium (m equiv)*	*Chloride (m equiv)*
1	growth hormone	12	+ 384	+ 252
	post treatment	16	− 32	− 64
		Total	+ 352	+ 188
2	growth hormone	16	+ 320	+ 144
	post treatment	12	− 288	− 264
		Total	+ 32	− 120
3	growth hormone	14	+ 224	+ 175
4	growth hormone	14	+ 252	+ 196

Table VII

VOLUME OF EXTRACELLULAR FLUID

Period	*Days*	*Extracellular fluid (l)* case 1	case 2	case 3	case 4
control	− 8	18 5	28 9	17 [illegible]	18 2
	− 1	18 2		17 9	18 4
growth hormone	+ 7	19 0	30 0	17 7	18 9
	+ 14	18 6*	30 7	18 3	19 9
post treatment	+ 21	17 7	29 9		
	+ 28	18 8	27 4		

Two days after discontinuation of growth hormone administr[illegible]

Carbohydrate metabolism and ketogenesis

The significance of growth hormone in carbohydrate metabolism and ketogenesis since it was first demonstrated by the pioneer work of Houssay and Young has been well established One of the main objects of the present study was therefore to examine the effect of human growth hormone on these metabolic processes In the first series of studies human growth hormone was given to three patients with juvenile diabetes mellitus who had been hypophysectomized a few years earlier because of rapidly progressing vascular complications (Luft *et al* 1958 1959) The use of such subjects was suggested by the fact that the diabetogenic effect of growth hormone is most pronounced in diabetic animals especially if they have been hypophysectomized previously (see the review by de Bodo and Altszuler 1957)

The effect of a daily dose of 10 mg of human growth hormone on the three hypophysectomized juvenile diabetics can be inferred from Fig 3 In the first of them the administration of growth hormone was followed by a prompt deterioration in the diabetic state as shown by a rise in the blood sugar level in glycosuria and ketonuria (Fig *3a*) The general condition of the patient deteriorated rapidly and the study therefore had to be discontinued 36 hours later by giving the patient a large extra dose of insulin The response of the second patient to growth hormone was identical with that of the first one Here too a large dose of extra insulin had to be given 36 hours after the first injection of growth hormone (Fig *3b*) The response of the third patient was qualitatively identical with that of the previous two (Fig *3c*) However in this case the deterioration in the diabetic state was slower and it was possible to continue administration of growth hormone for eight days Furthermore in this case the diabetic state returned slowly to the pre treatment condition, and the ketonuria decreased to the initial level without extra insulin being given We have no data regarding the nitrogen balance in these patients

All these three patients got growth hormone from the same

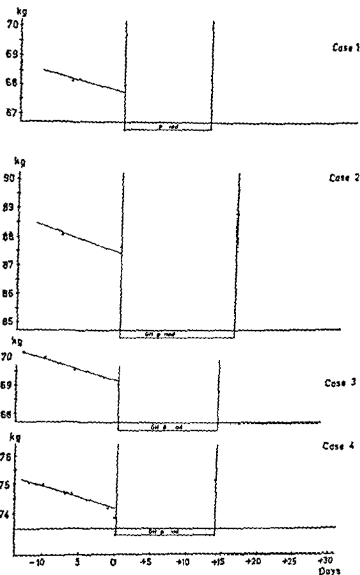


FIG 2 Changes in body weight

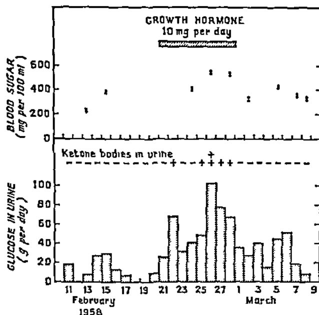


FIG 3c

FIG 3 *a b* and *c* Effect of growth hormone on the diabetic state in three hypophysectomized juvenile diabetics (From Luft *et al* 19 8 Reproduced by permis ion of the Editors *Lancet*)

batch We later had the opportunity of studying the effect of two other batches of human growth hormone on Case 2 and almost identical results were obtained The only difference hitherto seen was that one of the preparations had a much more prolonged effect and extra insulin up to 60 units per day (ordinary daily insulin requirement 16 units) had to be given for 10 days after a single injection of 10 mg of growth hormone

The ketogenic effect of growth hormone in these patients is even more striking This is because metabolic acidosis seldom if ever develops in hypophysectomized diabetic patients under conditions which in the presence of the hypophysis would lead to ketosis In four hypophysectomized diabetic subjects insulin was withdrawn for 24–48 hours and despite

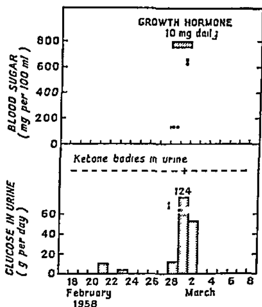

GROWTH HORMONE
10 mg daily
BLOOD SUGAR
(mg per 100 ml)
800
600
400
200
0
Ketone bodies in urine
GLUCOSE IN URINE
(g per day)
124
60
40
20
0
18 20 22 24 26 28 2 4 6 8
February
1958
March

FIG 3a

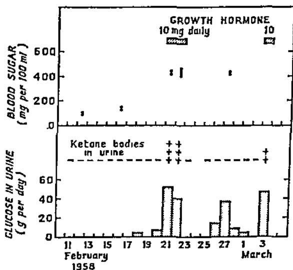

GROWTH HORMONE
10 mg daily
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Ketone bodies
in urine
GLUCOSE IN URINE
(g per day)
60
40
20
0
11 13 15 17 19 21 23 25 27 1 3
February
1958
March

FIG 3b

the intact patients reacted so dramatically to growth hormone as the hypophysectomized ones although the former received daily doses of growth hormone three times as large It should be added that the growth hormone given to the intact patients had been tested for activity in the above hypophysectomized Case 2

This absence of a striking effect of growth hormone in non hypophysectomized diabetics was unexpected in the light of experience from animal studies No explanation of it can be given at present

The effect of growth hormone on carbohydrate metabolism and ketogenesis has also been studied in non diabetic subjects Fasting blood sugar level glycosuria and ketonuria were followed in all four patients in the aforementioned metabolic study, who received 10 mg of growth hormone daily Fasting blood sugar remained unchanged and glycosuria did not appear during growth hormone treatment On the other hand, ketonuria increased in all but one of the patients (Fig 4) This increase was noted within 48 hours of the first injection but later decreased despite the continuation of growth hormone Intravenous glucose tolerance tests in two of these subjects showed a decreased disappearance rate of glucose (k value) which persisted during the whole treatment period (Fig 5)

In the next series eleven non diabetic subjects without any obvious disease were given intramuscularly 30 mg of growth hormone daily in divided doses (15 mg every 12 hours or 10 mg every 8 hours) for three days (Ikkos Luft and Gemzell 1959b) All subjects were given a high carbohydrate diet—300 g or more of carbohydrate daily—for at least 4 days before growth hormone administration and during it Immediately before the first growth hormone injection as well as the day after the last injection (8–12 hrs after it) a modified intravenous glucose tolerance test was performed in the morning in a post absorptive state as follows after a priming intravenous injection of 100 ml of 25 per cent glucose solution a constant infusion of a 15 per cent glucose solution was given

the marked increase in hyperglycaemia there was only a minor increase in ketone body excretion

The question then arose whether this pronounced effect of growth hormone in hypophysectomized juvenile diabetics was due to hypophysectomy or to the type of diabetes Therefore in the next series of studies growth hormone was given to non hypophysectomized patients with diabetes of either the juvenile or mature onset type (Luft *et al* 1959) The results so far obtained are presented in Table IX Because of the

Table IX

EFFECT OF GROWTH HORMONE ON THE DIABETIC STATE IN HUMAN DIABETIC SUBJECTS

		Growth hormone (10 mg /day) Increase in			*Growth hormone (30 mg /day) Increase in*		
	Case	*blood sugar*	*glycos uria*	*keton uria†*	*blood sugar*	*glycos uria*	*keton uria†*
Hypophysec tomized juvenile	K E B	+	+	+			
	A G	+	+	+			
	B B	+	+	+			
	G O	+	+	+			
Non hypo physectom ized juvenile	T K	—	—	—	+	+	—
	B A	—	—	—	+	+	+
	L C	—	—	—			
	R M S				+	+	(+)
Mature onset on insulin	O C				—	—	—
	B G	(+)	(+)	—	+	+	—
Mature onset on oral antidiabetic drugs	G J	—	—	—			
	A F				+	+	—
	K E J				—	—	—
	C B				+	+	(+)
	K D				+	+	—

— denotes no change (+) a probable increase and + an unequivocal increase in blood sugar or glycosuria
† — denotes no change (+) an increas but within the limits observed in non-diabetic subjects on growth hormone and + a pronounced increase in ketonuria

small number of patients no definite conclusions can be drawn There did not however seem to be any difference between the two groups of diabetics with respect to their reaction to growth hormone Moreover the absence of the pituitary gland increases the sensitivity to growth hormone since none of

the intact patients reacted so dramatically to growth hormone as the hypophysectomized ones although the former received daily doses of growth hormone three times as large It should be added that the growth hormone given to the intact patients had been tested for activity in the above hypophysectomized Case 2

This absence of a striking effect of growth hormone in non hypophysectomized diabetics was unexpected in the light of experience from animal studies No explanation of it can be given at present

The effect of growth hormone on carbohydrate metabolism and ketogenesis has also been studied in non diabetic subjects Fasting blood sugar level glycosuria and ketonuria were followed in all four patients in the aforementioned metabolic study who received 10 mg of growth hormone daily Fasting blood sugar remained unchanged and glycosuria did not appear during growth hormone treatment On the other hand, ketonuria increased in all but one of the patients (Fig 4) This increase was noted within 48 hours of the first injection but later decreased despite the continuation of growth hormone Intravenous glucose tolerance tests in two of these subjects showed a decreased disappearance rate of glucose (k value) which persisted during the whole treatment period (Fig 5)

In the next series eleven non diabetic subjects without any obvious disease were given intramuscularly 30 mg of growth hormone daily in divided doses (15 mg every 12 hours or 10 mg every 8 hours) for three days (Ikkos Luft and Gemzell, 1959*b*) All subjects were given a high carbohydrate diet—300 g or more of carbohydrate daily—for at least 4 days before growth hormone administration and during it Immediately before the first growth hormone injection as well as the day after the last injection (8–12 hrs after it) a modified intravenous glucose tolerance test was performed in the morning in a post absorptive state as follows after a priming intravenous injection of 100 ml of 25 per cent glucose solution a constant infusion of a 15 per cent glucose solution was given

the marked increase in hyperglycaemia there was only a minor increase in ketone body excretion

The question then arose whether this pronounced effect of growth hormone in hypophysectomized juvenile diabetics was due to hypophysectomy or to the type of diabetes Therefore, in the next series of studies growth hormone was given to non hypophysectomized patients with diabetes of either the juvenile or mature onset type (Luft *et al* 1959) The results so far obtained are presented in Table IX Because of the

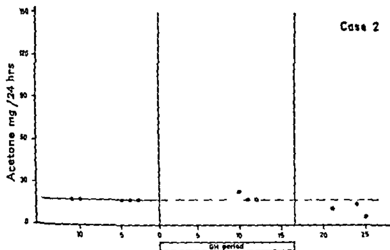
Table IX

EFFECT OF GROWTH HORMONE ON THE DIABETIC STATE IN HUMAN DIABETIC SUBJECTS

		Growth hormone (10 mg /day) Increase in			*Growth hormone (30 mg /day) Increase in*		
	Case	*blood sugar*	*glycosuria*	*ketonuria†*	*blood sugar*	*glycosuria*	*ketonuria†*
Hypophysectomized juvenile	K E B	+	+	+			
	A G	+	+	+			
	B B	+	+	+			
	G O	+	+	+			
Non hypophysectomized juvenile	T K	—	—	—	+	+	—
	B A	—	—	—	+	+	+
	L E	—	—	—			
	R M S				+	+	(+)
Mature onset on insulin	O D				—	—	—
	B G	(+)	(+)	—	+	+	—
Mature onset on oral antidiabetic drugs	G J	—	—	—			
	A F				+	+	—
	K E J				—	—	—
	C B				+	+	(+)
	K D				+	+	—

— denotes no change (+) a probable increase and + an unequivocal increase in blood sugar or glycosuria
† — denotes no change (+) an increase but within the limits observed in non diabetic subjects on growth hormone and + a pronounced increase in ketonuria

small number of patients no definite conclusions can be drawn *There did not however seem to be any difference between the* two groups of diabetics with respect to their reaction to growth hormone Moreover the absence of the pituitary gland increases the sensitivity to growth hormone since none of



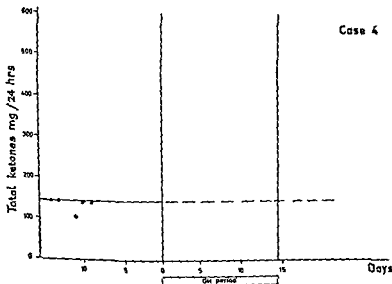


Fig 4b Urinary excretion of ketone bodies

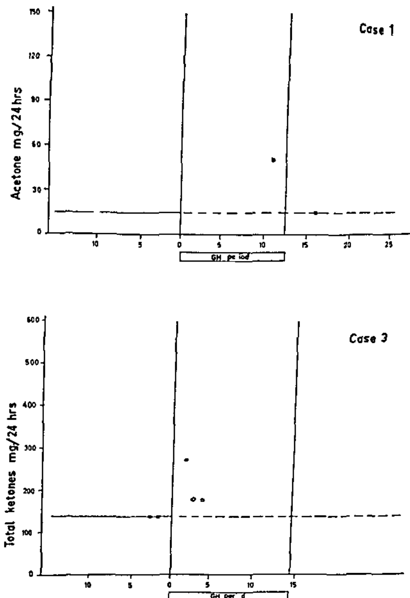


FIG 4a Urinary excretion of ketone bodies

cases but decreased in the remaining seven In six of these seven cases the k value was 1 0 or less after treatment with growth hormone (Fig 6) The changes in the concentration of pyruvic lactic citric and α ketoglutaric acids during the

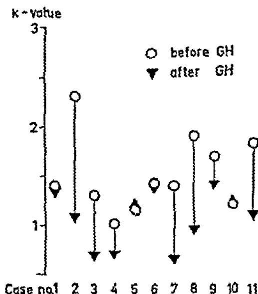


FIG 6 Changes in the rate of disappearance of glucose (k value) with 30 mg of growth hormone per day

glucose tolerance test (Fig 7) were uninfluenced by the growth hormone treatment except in those three cases (nos 3 4 and 7) in which the lowest values for the rate of disappearance of glucose were observed after growth hormone (0 68 0 70 and 0 64 respectively) The non esterified fatty acid level in plasma was increased by growth hormone but the response of non esterified fatty acid to intravenous glucose was not changed by growth hormone (Fig 8)

Thus growth hormone in a daily dose of 10–30 mg in short term experiments induced a decrease in the rate of dis appearance of glucose in 9 out of 13 non diabetic subjects

for one hour at a rate of 8 2 ml /min (about 30 g of glucose) Blood glucose concentration was measured before the test at the end of the infusion and every 5 minutes thereafter for one hour The rate of disappearance of glucose (k value) after discontinuation of the infusion was calculated by plotting on a semilog paper the blood glucose concentration against time

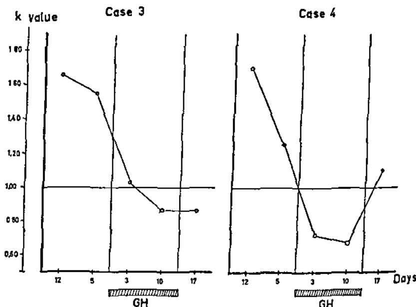


FIG 5 Changes in the rate of disappearance of glucose (k value) with 10 mg of growth hormone per day

During these tests the concentration of pyruvic acid lactic acid citric acid and α ketoglutaric acid as well as of non esterified fatty acids and inorganic phosphate was followed in the peripheral blood Venous blood samples for determination of these metabolites were taken immediately before the priming injection (0 min) immediately upon discontinuation of the infusion (60 min) and one hour later (120 min)

The results can be summarized as follows The rate of disappearance of glucose (k value) remained unchanged in four

latter finding were to be valid in man as well then the de creased rate of disappearance of glucose could be a sign of increased glucose production This problem is now being investigated with ^{14}C glucose

Significant changes in the concentration of intermediary metabolites were observed only in 3 out of 11 subjects given

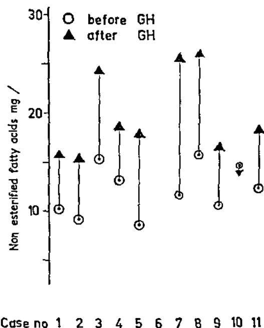


FIG 8 Changes in non esterified fatty acids in plasma

30 mg of growth hormone although the k value was influenced in 7 of them This inconsistency cannot be ascribed to dif ferences in the preparations of growth hormone Two prep arations were used in these 11 subjects and both were found to be active increases in non esterified fatty acids were observed with both preparations as seen in Fig 8 Further more both preparations had a pronounced diabetogenic action

Our results are therefore similar in this respect to the findings of others (Beck *et al* 1958) The significance of this finding cannot be evaluated at present One simple explanation would be that this decrease was brought about by a decreased

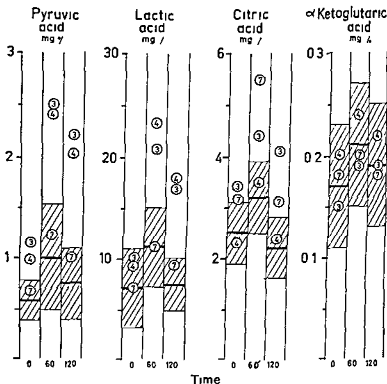


FIG 7 Effect of growth hormone on the changes in some metabolites during acute glucose loading in 11 subjects Shaded areas denote ±2 range before growth hormone In cases not plotted the values obtained with growth hormone were within the pretreatment limits (Figures within circles denote case numbers)

peripheral utilization of glucose i e that growth hormone induced a 'pre diabetic state However despite the fact that growth hormone causes hyperglycaemia and a diabetic glucose tolerance in dogs the peripheral utilization of glucose increases with growth hormone (Altszuler *et al* 1959) If this

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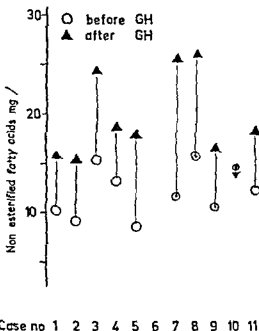


FIG 8 Changes in non-esterified fatty acids in plasma

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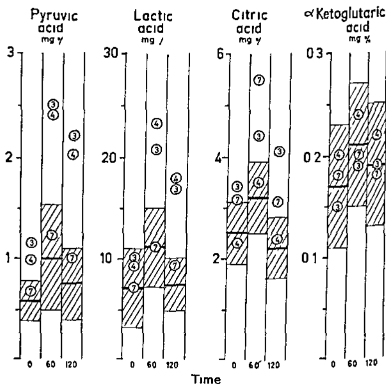


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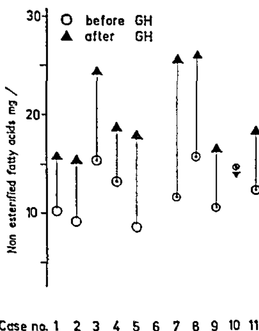


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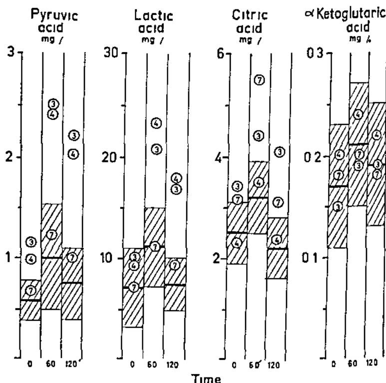


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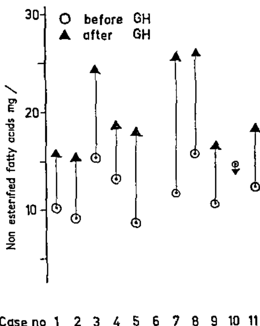


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in a hypophysectomized diabetic subject (the patient in Fig 4) A fact which may be of importance in this connexion is that the three cases with changes in the intermediary metabolites were those in which the lowest k values were noted after growth hormone It might well be that the metabolic effect must be pronounced before changes in the concentration of intermediary metabolites in the peripheral blood can be measured

An excessive increase in pyruvate and lactate during a glucose load was reported by Henneman and Bunker (1957) in patients with elevated 17 hydrocorticoids in blood It is improbable however that our results were due to contamination of the growth hormone with ACTH since the changes in citrate concentration after growth hormone were the opposite of those seen with cortisone (Henneman and Henneman 1958)

Our data are too limited to allow any definite conclusion regarding the mechanism of the changes observed They are the reverse of those that can be expected from insulin deficiency where the increase in pyruvate and lactate upon glucose loading is small or absent The increase in pyruvate, lactate and citrate in our three subjects might be due to an overloading of the common metabolic pathway from increased catabolism of fat That fat catabolism increases on growth hormone administration is obvious even from the present results showing an increase in non esterified fatty acids and ketone body excretion We are now making further studies of this mechanism as well as of other mechanisms conceivably responsible for the changes in question

REFERENCES

ALTSZULER N STEELE R WALL J S DUNN A and DE BODO R C (1959) *Amer J Physiol* 196 121

ASTWOOD E B (1955) *In* The Hormones Physiology Chemistry and Applications vol 3 235 eds Pincus G and Thimann K V New York Academic Press

BECK J C MCGARRY E E DYRENFURTH I and VENNING E H (1958) *Arch intern Med* 49 1090

DE BODO R C and ALTSZULER N (1957) *Vitam and Horm* 15 205

EINHORN J (1958) *Acta radiol (Stockh)* Suppl. 160
HENNEMAN D H. and BUNKER, J P (1957) *Amer J Med* 23 35
HENNEMAN D H. and HENNEMAN P H (1958) *J clin Endocr* 18 1093
HOKFELT B IKKOS D., and LUFT R (1959) Unpublished data.
IKKOS D LUFT R. and GEMZELL, C. A. (1958) *Lancet* i, 720
IKKOS D LUFT R and GEMZELL, C A. (1959a) *Acta endocr (Kbh)* 32 141
IKKOS D LUFT R and GEMZELL, C A (1959b) Unpublished data.
LI C H and PAPKOFF H (1956) *Science* 124 1293
LUFT R IKKOS D and GEMZELL, C A. (1959) Unpublished data.
LUFT R. IKKOS D GEMZELL, C A. and OLIVECRONA, H (1958). *Lancet* i 721
LUFT R. IKKOS D GEMZELL, C A. and OLIVECRONA, H. (1959) *Acta endocr (Kbh)* 32 330
Panel appointed by The Clinical Endocrinology Committee of the Medical Research Council (1959) *Lancet* i, 7
SMITH R W Jr GAEBLER, O H and LONG C. N H Eds (1955) The Hypophyseal Growth Hormone Nature and Actions. New York McGraw Hill
WILHELMI A E (1955) *In* The Hypophyseal Growth Hormone Nature and Actions p 59 eds Smith, R W Jr Gaebler O H., and Long C N H New York McGraw Hill.

DISCUSSION

Pearson Dr Luft is to be congratulated on his excellent metabolic studies on the effects of human growth hormone Our experience has been very similar to his A most interesting aspect is the markedly different effects of growth hormone in the non-diabetic versus the diabetic patient We have worked with human growth hormone prepared by Raben using doses of 5 instead of 10 mg per day as Dr Luft has used We have found that in non-diabetic hypophysectomized patients 5 mg of growth hormone daily produces very marked anabolic effects but essentially no diabetogenic effects whereas in the hypophysectomized diabetic patient 5 mg of growth hormone rarely produces anabolic effects but usually produces very marked diabetogenic effects In the juvenile type of diabetic after hypophysectomy a single injection of 5 mg of growth hormone produces severe ketosis acidosis and negative nitrogen balance which is quite different from the response of the non-diabetic hypophysectomized individual We have studied the effects of growth hormone both before and after hypophysectomy in a patient with juvenile type diabetes A 30 year-old male with a very brittle type of diabetes was maintained on a constant diet and constant insulin dosage He was given growth hormone for three days at a dose of

5 mg per day It had very little effect on the blood sugar or on the urinary excretion of glucose There was a definite rise in urinary ketone excretion but no significant acidosis developed After hypophysectomy under a similar regimen, a single injection of 5 mg of human growth hormone induced profound hyperglycaemia glycosuria ketonuria and metabolic acidosis within 24 hours The patient became quite ill but spontaneous recovery occurred without the addition of extra insulin We have observed similar profound diabetogenic responses induced by small doses of human growth hormone in other hypophysectomized juvenile type diabetic patients The contrast in the response to growth hormone before and after hypophysectomy is extremely interesting and in accord with the results that Dr Luft has presented comparing non hypophysectomized with hypophysectomized diabetic patients It would appear that withdrawal of growth hormone by hypophysectomy is responsible for the amelioration of the diabetes in these patients This raises the question whether withdrawal of growth hormone is responsible for the improvement of diabetic retinopathy which may be seen after hypophysectomy

The effects of human growth hormone before and after hypophysectomy have also been studied in patients with a milder adult type of diabetes Human growth hormone in doses of 5–10 mg per day for several days induced hyperglycaemia glycosuria and ketonuria but without significant acidosis The response may be somewhat greater after hypophysectomy than before but severe acidosis with these doses of growth hormone was not encountered Thus the diabetogenic effects of growth hormone appear to be related to the apparent ability of the patient s pancreas to release insulin

Luft I mentioned in my review that we had not measured the nitrogen balance in any of the three hypophysectomized diabetic patients who were given growth hormone We intended to do such measurements in all three of them but as you noticed we had to stop the administration of growth hormone after two injections in two of them In the third patient who was given growth hormone for eight days we measured the nitrogen excretion However the daily fluctuations in urinary nitrogen were too large to allow calculation of the balance with any degree of certainty As far as we could see there was no definite change in nitrogen balance during the growth hormone period

Venning Dr Luft by what method and in what fraction were you measuring the aldosterone ?

Luft The measurements of aldosterone in urine were performed by Dr B M Hokfelt in our department and he is using a modification of the Neher Wettstein method

Venning Have you had any opportunity of measuring the reduced metabolite of aldosterone?

Luft No that has not been done

Venning We have measured in two patients with diabetes the excretion of the reduced metabolite of aldosterone before and after the administration of 5 mg of growth hormone daily and we found that while there was no significant increase in the acid released fraction of aldosterone there was a significant rise in the reduced metabolite

Luft This is very interesting Dr Venning and we certainly must repeat your studies Would you infer from your data that this increase in aldosterone might explain the sodium retention during growth hormone administration?

Venning It is possible that these changes may not be entirely dependent upon variations in aldosterone secretion Our studies show that one may miss this rise in aldosterone by only measuring the free fraction

Beck Dr Luft can you tell us what was the phosphate intake of the three patients out of the four that you have studied in whom there was a rise in both urinary and faecal calcium?

Luft I have no data at hand regarding the phosphate intake but I would call it a normal one

Beck We have done some work on arteriovenous glucose differences in relation to growth hormone and in two studies there is a suggestive decrease in arteriovenous or capillary venous differences which suggests a decrease of peripheral utilization when diabetogenicity is present The problem of inducing anabolism and glycosuria in diabetics is one that we must look upon as graded from those in which anabolism occurs with minor impairment of carbohydrate tolerance to those in which no anabolism occurs with very marked intensification of hyperglycaemia Both our own results and some that Dr Dorothy Henneman obtained when she worked in our laboratory would suggest this

de Ulhôa Cintra Dr Luft did you notice any change from normal in the serum phosphate in diabetic patients?

Luft The blood level of inorganic phosphate is of special interest in studies with growth hormone since acromegalic patients show an increase in this electrolyte and we use plasma phosphate in these patients as an indicator of the activity of the disease However we could not observe a significant change in plasma phosphate in any of the patients during the relatively short periods of growth hormone administration from 12–16 days We were a little surprised at this negative observation but it may well be that we have to give growth hormone for a much longer period of time in order to obtain an

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Venning Dr Luft by what method and in what fraction were you measuring the aldosterone ?

Luft The measurements of aldosterone in urine were performed by Dr B M Hokfelt in our department and he is using a modification of the Neher Wettstein method

the patients but it could be that two daily doses give a better response than one

Li There has been some indication from our unpublished animal experiments that human growth hormone divided into three daily doses gives a better response in the tibia test than it does when it is administered only once daily whereas with beef growth hormone there is no difference in response

Luft If the half life of human growth hormone is nine hours as Dr Gemzell mentioned we did the right thing when we divided the daily dose of growth hormone into two injections

Venning Dr Luft have you any ideas regarding the mechanism of increased sensitivity of the hypophysectomized patient to growth hormone?

Luft This question is difficult to answer It is well known that the hypophysectomized patient or the patient with panhypopituitarism is much more sensitive to thyroid hormone or cortisone than the patient with an intact pituitary The explanation for the increased sensitivity to growth hormone may well be that hypophysectomized patients are lacking in growth hormone

Pearson There is fairly good evidence that the adult individual produces growth hormone and that this is circulating Daughaday and co workers have assayed a sulphation factor in human plasma which declines to very low levels after hypophysectomy and may represent a measure of growth hormone levels (Daughaday W H Salmon W D Jr and Alexander F (1959) *J clin Endocr* **19** 743) If it is assumed that the diabetic is producing growth hormone and if you now add an increment of exogenous growth hormone this might have much less effect than if there was no growth hormone at all and then you administered some This may not be the answer but it seems to be a possible explanation

de Ulhoa Cintra Dr Luft have you any experience of the k index in other conditions such as Cushing s or Addison s disease (the k value in intravenous glucose tolerance tests)?

Luft The intravenous glucose tolerance test is used routinely in my department and we always calculate the disappearance rate of glucose i e the k value using total glucose and not excess glucose We have noticed decreased k values which means a diabetic type of curve in patients with Cushing s syndrome as well as in acromegalic patients As far as I can recall our patients with Addison s disease had normal k values I think the data on patients with acromegaly should be emphasized in this respect In contrast to statements in the literature we seldom see overt diabetes in acromegaly as a matter of fact we have noticed it in only one out of about 50 patients On the other hand a lowered disappearance rate of glucose has been

increase in plasma phosphate I may also add that neither did we get any increase in alkaline phosphatase

Raben It is likely that the effects on serum phosphorus and on alkaline phosphatase are slow to appear, but both increase during prolonged treatment of pituitary dwarfs with human growth hormone In one patient whom I have treated for twenty seven months with 2 mg of growth hormone three times a week i e about 1 mg a day, serum phosphorus was 3 6 mg per cent at the beginning and after some months it was 4 8 then 6 and the most recent value was 6 3 mg per cent Alkaline phosphatase, which was 4 2 Bodansky units initially increased to 6 6 with treatment The growth rate was 2 5 inches per year which is slightly greater than the normal rate

A 17 year old pituitary dwarf grew 2 25 inches in 6 months on treatment with the same dose i e 2 mg of growth hormone 3 times a week and his serum phosphorus rose from 4 2 to 6 6 mg per cent and phosphatase from 4 2 to 7 Bodansky units In both these cases the blood sugar did not change with treatment The patients were treated with thyroid and cortisone for 18 months in one case and 8 months in the other before receiving growth hormone and these hormones were continued during the growth hormone treatment

Russell Fraser Have you ever produced growth without adding thyroid to your hypopituitary patients ?

Raben In each case the patient continued to receive the same amount of thyroid as before growth hormone treatment

Beck We have tried by accident Dr Raben and we can produce growth in the absence of thyroid hormone in a hypopituitary dwarf However it should be pointed out that these people are not athyrotic — the protein bound iodine was around 2—but there was quite definite growth and it did not seem to be different from when they were receiving adequate amounts of thyroid We also have noticed that in prolonged studies the serum alkaline phosphatase and the serum phosphorus is elevated we only get a relatively small change during acute administrations

Li Dr Luft you mentioned that in the last three patients from whom you quoted data you injected growth hormone three times a day instead of once a day In view of the data which we heard earlier indicating that growth hormone apparently has a long biological half life is it really necessary to inject three times a day or is one daily dose sufficient ?

Luft Before starting the experiment we discussed this point and decided that it might be safer to divide the daily dose into two injections especially since we were giving the patients a rather large amount of growth hormone Besides it could not in any way harm

THE EFFECT OF GROWTH HORMONE ON URINARY CALCIUM EXCRETION

Russell Fraser and Michael Harrison

Department of Medicine Postgraduate Medical School of London

It is appropriate that a colloquium in Buenos Aires should review the subject of the effect of growth hormone on urinary calcium Professor Houssay among his extensive studies and publications about the anterior pituitary gave in 1936 the Harvey lectures on the subject of the association between the anterior pituitary the parathyroids and the pancreatic islets In this lecture he reviewed work of his own and others indicating a close influence of the anterior pituitary on pancreatic islet function and also on parathyroid function Since then much work notably that contributed by Professor Houssay and Professor Young has led to the general acceptance of a close inter relationship between the growth hormone and the islets of Langerhans Pancreatic diabetes is ameliorated by hypophysectomy while growth hormone injections induce islet hypertrophy in young animals and after prolonged administration islet exhaustion Growth hormone however is not regarded as a pancreatotropic hormone i e one having a direct effect on the pancreas like that of thyrotropin on the thyroid but rather as increasing secretory activity in the islets by indirect means Both before and since Professor Houssay s lecture various authors have postulated a rather similar relationship between the anterior pituitary and the para thyroid glands but possibly because these small parathyroid glands are so difficult to study in the usual experimental animals no final conclusions have been reached Several authors have failed to see a stimulating effect on the para thyroids following either anterior pituitary injections or growth hormone injections and therefore have concluded that

a common finding in this group of patients Dr Ikkos and I are now studying glucose metabolism in acromegaly in the same way that has been demonstrated in my presentation today

de Ulhoa Cintra The variations in healthy people are small?

Luft The variations in healthy subjects are not very large I may also mention that the lower normal limit for λ seems to be about 1

Pearson Dr Luft have you studied any other pituitary hormones for their ketogenic or fat mobilizing effects in your diabetic patients? For example do ACTH TSH gonadotropins and so forth have any effect? I am thinking in terms of the animal studies of Engel which indicated that ACTH may have extra adrenal effects on fat mobilization (Engel F L and Engel M G (1958) *Endocrinology* 62 150)

Luft We have not done this systematically Dr Pearson In some hypophysectomized diabetic patients when given insulin thyroid extract and cortisone we increased the daily dose of cortisone to about 200–300 mg per day without getting any marked increase in ketone body excretion however hyperglycaemia and glycosuria increased These studies should be repeated with ACTH but it might also be a good idea to study the effect of large doses of ACTH in adrenalectomized diabetic patients if such are still available

Gomez Mont Did the hypophysectomized patients receive thyroid medication at the time when they received growth hormone?

Luft All hypophysectomized patients given growth hormone had a daily dose of insulin cortisone and thyroid extract which was the same during the control period and—except for insulin—the period of growth hormone administration

Gomez Mont Would there have been any difference if you had not given thyroid to these patients?

Luft That I cannot say but I doubt it

Russell Fraser I don t know that I could answer that entirely but certainly our hypophysectomized patients given growth hormone without supporting thyroid therapy show a mild degree of diabetogenic effect on their fasting blood sugar Our group has studied eight patients and perhaps two of them were not having thyroid

Sonenberg Have Dr Luft or Dr Gemzell any information on the difference in the half life of growth hormone in normal and in hypophysectomized patients?

Gemzell I could find no difference We studied two non hypophysectomized and four hypophysectomized patients but as I said earlier the value we obtained was not significant and to get the half life we had to combine all the values

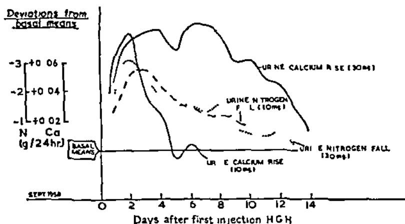


Fig 1 Mean urinary nitrogen and calcium changes after 10 mg or 30 mg HGH in hypopituitarism (10 mg to 2 patients and 30 mg to 4 patients) (Reproduced by permission of the Editors *Lancet*)

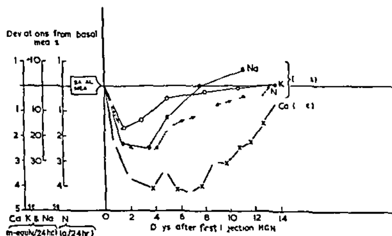


Fig 2 Mean changes in urinary nitrogen potassium sodium and calcium after 30 mg HGH in 4 hypopituitary patients (Reproduced by permission of the Editors *Lancet*)

a parathyrotropic effect of growth hormone is unlikely but others have claimed the opposite and so an indirect effect remains possible but unproved

The recent revival of interest in the human response to growth hormone, arising from Li and Papkoff's demonstration (1956) of the difference between human growth hormone (HGH) and bovine growth hormone (BH) will be familiar to this audience Several authors have observed nitrogen retention and other effects when HGH was given to man We have also observed in man similar strong anabolic effects from HGH administration, along with several colleagues in a study co ordinated through the clinical endocrinology committee of the Medical Research Council (1959) In our patients perhaps the most striking metabolic effect was the increased urinary calcium loss A similar calciuric effect was also observed by most of the other authors on giving HGH to man Most of them however found an associated decreased faecal loss of calcium and so a net retention of calcium Consequently this hypercalciuria has not received much attention However this compensatory increased intestinal absorption of calcium was not seen in many of our patients and even so the mechanism of the calciuria remains a problem It immediately recalls the experiments such as those reviewed by Houssay in 1936 and also the problem of osteoporosis in acromegaly

Calciuria following HGH administration to man

Fig 1 will recall the effects we observed using two dose levels in hypopituitary patients after 10 mg a moderately intense calciuria lasting about 7 days and after 30 mg an equivalent initial calciuria lasting longer and evident for 14–21 days By contrast there was little difference between the two dose levels in their urinary nitrogen effects This suggests that the calciuric effect might be an effect of growth hormone excess rather than of growth hormone replacement in a hypopituitary subject a point also suggested by our finding this effect even in the two normal subjects tested It may be noted

serum calcium and phosphate suggests the sudden onset of osteoporosis One patient showed this calcium effect much less than the others but the only difference in his regime seemed to be a much lower caloric intake These patients showed little change in serum or urinary phosphate (P) following the growth hormone (Fig 4) A slight fall in urinary

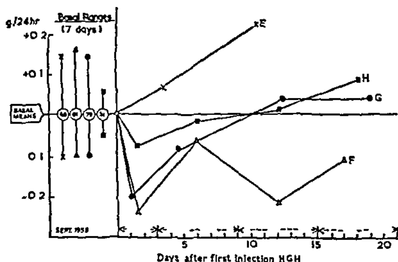


FIG 4 Urinary phosphate changes after 80 mg HGH in 4 hypopituitary patients (Reproduced by permission of the Editors *Lancet*)

phosphate was observed but this was found to correspond to the theoretical P change calculable from calcium and nitrogen balances i e what would be expected from some phosphate being deposited with the nitrogen in the tissues and some being released with the calcium from the bones

Confirmation in rats (Figs 5 and 6)

To study this calciuric effect further it seemed important to establish first whether it could be observed in other species using growth hormone from other sources To this end

that the duration of the calcium effect after 30 mg HGH corresponded to that of the urinary nitrogen effect, suggesting that it also reflected the same metabolic change induced by the growth hormone and not due to impurities (Fig 2) By contrast, while the phosphorus also followed the nitrogen retention the sodium retention observed was of much shorter duration, other reasons, nevertheless, suggest that this latter is also a true HGH effect Further, these patients were found to

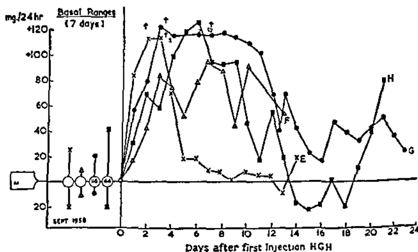


FIG 3 Urinary calcium changes after 30 mg HGH in 4 hypopituitary patients (Reproduced by permission of the Editors *Lancet*)

show no signs of thyroid stimulation following this growth hormone administration and so a thyrotropic effect was an unlikely explanation for this change in calcium metabolism One patient (Fig 3) was on a low calcium diet and in this patient the calcium could only have come from the bones the extent of the calcium effect was also equivalent to that in the other patients In addition our data showed no change in serum calcium or phosphate suggesting therefore a change in renal clearance of calcium possibly due to a change in its mode of carriage in the blood Such a hypercalciuria with normal

doses of growth hormone were large (3 mg daily for three doses) since the human experiments suggested that this hypercalciuria may be due to overdosage rather than to a simple replacement effect of growth hormone

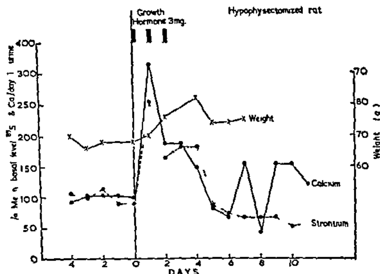


FIG 6 Comparison of 85Sr and calcium excretion in urine following injection of GH in hypophysectomized rats.

Studies to assess the mechanism of the calciuria (Fig 7)

Our next experiment was to repeat the observations recorded above under similar conditions in rats which were parathyroidectomized as well as hypophysectomized Confirmation of the parathyroidectomy was obtained from the final blood levels of calcium and phosphate It will be observed (Fig 7) that in contrast with the previous rats these parathyroidectomized rats showed no calciuric response to growth hormone both the strontium and the calcium excretions continuing the same as during the preliminary basal period

therefore we put some young rats on a low calcium diet and injected them with radioactive strontium (^{89}Sr intraperitoneally) to label their skeletons, at least 16 days were allowed for equilibration of the strontium throughout their bones, it was thus possible to follow the urinary calcium and ^{89}Sr excretion without contamination by food. We should then have two indices of any calciuric effect of the growth

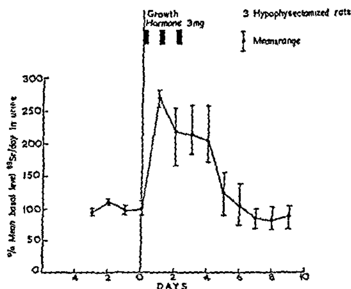


Fig 5 ^{89}Sr excretion in urine following injection of GH in hypophysectomized rats

hormone the excreted calcium and the excreted strontium. When little effect was observed on these excretions in the normal rats given large doses of growth hormone, the same experimental procedure was carried out in hypophysectomized rats. Figs 5 and 6 show the results of these experiments, a striking rise in the excretion of calcium and strontium lasting for several days in a pattern reminiscent of the effects of HGH in man. The weight increase of the rats confirmed that the growth hormone was also producing its anabolic effects. The

It is perhaps unlikely that the effect is a direct parathyro tropic action since our observations in humans suggested that these effects were overdosage effects rather than physio logical level effects Previous authors have postulated that the growth hormone might affect parathyroid activity by its influence on serum phosphate (Tornblom 1949) This is possible, but we feel it may be less likely since no changes in serum phosphate or calcium were observed in our patients when these marked calciuric effects were induced As with the pancreatic islets the growth hormone may modify para thyroid function indirectly perhaps through its effects on cell metabolism affecting intracellular electrolytes which may be the normal stimulus to parathyroid activity

Good support is available in the literature for the thesis that growth hormone is a stimulus to parathyroid activity Hous say (1936) found histological atrophy of the parathyroids in hypophysectomized dogs, and in many of them it was cor rected after injections of anterior pituitary extract (APE) though others have not seen this atrophy (Baker 1942) Parathyroid atrophy has been noted in human panhypo pituitarism (Castleman and Hertz 1939 Sheehan and Sum mers 1949) in one instance being associated with tetany and a low serum calcium (Rupp and Paschkis 1953) On the other hand enlarged parathyroids with or without tumours are frequently found in acromegaly (Erdheim 1903 Cushing and Davidoff 1927 Perlman 1944 Underdahl Woolner and Black 1953) in which condition osteoporosis is also frequent (Albright and Reifenstein 1948) and balances on a low cal cium diet show hypercalciuria (Bauer and Aub 1941 Al bright and Reifenstein 1948) as we have also found Here it may be noted that as in acromegaly calcium deficiency with a normal vitamin D intake is associated with parathyroid hypertrophy and the bone lesion of osteoporosis (Harrison and Fraser 1959) The joint disorder frequently seen with acromegaly as well as this osteoporosis may depend upon some effect of excess growth hormone on bone matrix (Waine Bennett and Bauer 1945 Kellgren Ball and Tutton 1952

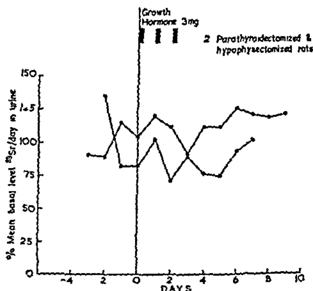


FIG 7 ⁸⁹Sr excretion in urine following injection of GH in hypophysectomized and parathyroidectomized rats

Discussion

We believe that the most likely explanation of these results is that these large doses of growth hormone stimulate parathyroid activity which they may do indirectly It is possible that the growth hormone may have acted on the bones only in association with a normal secretion rate of parathyroid hormone, but we think that this is less likely The parallel effects on calcium and strontium and their extent leave little doubt that the extra calcium must have been mobilized from the bones rather than by influencing the absorption of calcium from the gut The observation of this effect both with HGH in man and with BH in rats also makes it very likely that it is an effect truly attributable to growth hormone We have no further experiments of our own completed which can throw light on the mechanism by which the growth hormone might influence parathyroid activity

tumours without obvious acromegaly were subclinical examples of excess production of growth hormone like case J V with exophthalmos whom we have already discussed (this colloquium p 10) Indeed Albright and co workers (1934) considered previously whether their cases of hyperparathyroidism based on hyperplasia might not similarly arise from a primary pituitary stimulus

In this connexion we should also consider briefly what is the normal stimulus to parathyroid activity Much evidence points to its being a fall in serum or intracellular ionized calcium (Greep 1948) notably the finding of parathyroid hypertrophy in calcium or vitamin D deficiency However the opposite trends in phosphate concentration are also potent stimuli to the parathyroid and whether the calcium or the phosphate change is the final stimulus has been difficult to settle Many have accepted the experiments of Stoerk and Carnes (1945) as settling this in favour of calcium being the primary They measured parathyroid volume and the serum calcium and phosphate on groups of rats given diets ranging widely in regard to calcium and phosphorus content and concluded that as the parathyroid volume correlated most closely with serum calcium this was the primary stimulus Fig 8 has been drawn from their data and shows the groups ranged by parathyroid volume The serum calcium and phosphate seem to vary equally though inversely with parathyroid volume as also do the effective calcium and phosphorus in the diet It seems safest to conclude that either low calcium or high phosphate are effective stimuli to parathyroid activity As yet we can do little more than speculate on how growth hormone may stimulate the parathyroid Tornblom (1949) and Engfeldt (1950) concluded after reviewing many experiments that growth hormone stimulated the parathyroids by its tendency to raise serum phosphate incidentally Tornblom found that a high phosphorus and low calcium diet no longer stimulated the parathyroids in hypophysectomized animals

In conclusion we have available a feasible hypothesis that an excess of growth hormone may stimulate parathyroid

Asling *et al* 1955) In animals, APE has been found to produce parathyroid stimulation (Hertz and Kranes 1934 Ham and Haist, 1939, Blumenthal and Loeb 1942, Engfeldt 1950), though some have failed to observe this (Campbell and Turner, 1942) Generally no changes in serum calcium (Ca) have been seen with these various alterations of pituitary function though serum phosphate may rise with increased pituitary function

A relationship between pituitary and parathyroid function is also suggested by a recently established "pluriglandular' syndrome whose main manifestations are due to the co existence of hyperpituitarism (sometimes with acromegaly) hyperparathyroidism, and hyperinsulinism This association was first noted pathologically by Cushing and Davidoff (1927), first established with evidence of the triple disordered function and with a response to treatment by Shelburne and Mc Laughlin (1945) and first established as a syndrome by Under dahl Woolner and Black (1953) In a later review of 28 of these cases Moldawer Nardi and Raker (1954) concluded possibly incorrectly that the connecting link in the syndrome was not a functional interconnexion between these three and possibly other endocrine glands but instead a pluriglandular liability to tumours since some of the pituitary tumours were chromophobe and without evident acromegaly other tumours were also found at times in the adrenals pineal and in the bronchi and the condition was familial None of these arguments seem compelling in view both of the known tendency of growth hormone to stimulate the appearance of tumours in various sites and of the above physiological evidence that growth hormone can stimulate the pancreatic islets the parathyroids and probably also other endocrine glands Two interesting features of this syndrome are the adenomatous type of hyperplasia and the great frequency of multiple tumours in these affected glands and the rarity of the typical hyperparathyroid syndrome (bone disease and/or renal stones) despite the biochemical abnormalities It is quite probable that the patients with chromophobe pituitary

REFERENCES

Albright F Bloomberg E Castleman B and Churchill B D (1934) *Arch intern Med* **54** 315

Albright F and Reifenstein E C (1948) The Parathyroid Glands and Metabolic Bone Disease pp 188 190 Baltimore Williams & Wilkins

Asling C W Simpson M E Moon H D Li C H and Evans H M (1955) *In* The Hypophyseal Growth Hormone Nature and Actions p 154 eds Smith R W Jr Gaebler O H and Long C N H New York McGraw Hill

Baker B L (1942) *Anat Rec* **83** 47

Bauer W and Aub J C (1941) *J clin Invest* **20** 295

Blumenthal, H T and Loeb L (1942) *Endocrinology* **30** 502

Campbell I L and Turner C W (1942) *Res Bull Mo agric Exp Sta* No 352 134

Castleman B and Hertz S (1939) *Arch Path* **27** 69

Cushing H and Davidoff L M (1927) *Monogr Rockefeller Inst med Res* No 22

Engfeldt B (1950) *Acta endocr (Kbh)* suppl 6

Erdheim J (1903) *Beitr path Anat* **33** 158

Greep R O (1948) *In* The Hormones Physiology Chemistry and Applications 1 255 eds Pincus G and Thimann K V New York Academic Press

Ham A W and Haist R E (1939) *Nature (Lond)* **144** 835

Harrison M and Fraser R (1959) *J Endocr* **18** 25

Hertz S and Kranes A (1934) *Endocrinology* **18** 350

Houssay B A (1936) *Harvey Lect* **31** 116

Kellgren J H Ball J and Tutton G K (1952) *Quart J Med* **21** 405

Li C H and Papkoff H (1956) *Science* **124** 1293

Moldawer M P Nardi G L and Raker J W (1954) *Amer J med Sci* **228** 190

Panel appointed by the Clinical Endocrinology Committee of the Medical Research Council (1959) *Lancet* **i** 7

Perlman R M (1944) *J clin Endocr* **4** 483

Rupp J J and Paschkis K F (1953) *Ann intern Med* **39** 1103

Sheehan H L and Summers V K (1949) *Quart J Med* **18** 319

Shelburne S A and McLaughlin C W (1945) *J clin Endocr* **5** 232

Stoerk H C and Carnes W H (1945) *J Nutr* **29** 43

Tornblom N (1949) *Acta Endocr (Kbh)* suppl 4

Underdahl L O Woolner L B and Black B M (1953) *J clin Endocr* **13** 20

Waine H Bennett B A and Bauer W (1945) *Amer J med Sci* **209** 671

activity either by its tendency to raise phosphate levels in body fluids or by any other effect which would in turn lower the level of ionized calcium in the body fluids We have evidence that growth hormone may do this both acutely as

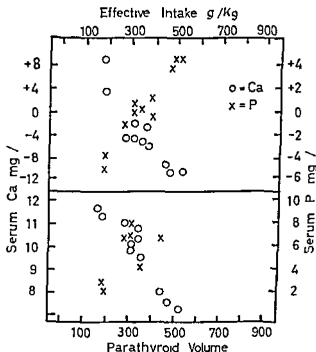


FIG 8 Rats on diets varying in calcium and phosphorus content parathyroid volume in relation to effective dietary and serum calcium and phosphorus (From data of Stoerk and Carnes 1945)

seen in our experiments and chronically as seen in acromegaly and in Underdahl s pluriglandular syndrome The biochemical hallmarks of this parathyroid stimulation by growth hormone are hypercalciuria with normocalcaemia together with mobilization of calcium from the bones which eventually leads to osteoporosis

REFERENCES

Albright F Bloomberg E Castleman B and Churchill, B D (1934) *Arch intern Med* 54 315

Albright F and Reifenstein E C (1948) The Parathyroid Glands and Metabolic Bone Disease pp 188 190 Baltimore Williams & Wilkins

Asling C W Simpson M E Moon H D Li C H and Evans H M (1955) *In* The Hypophyseal Growth Hormone Nature and Actions p 154 eds Smith R W Jr Gaebler O H and Long C N H New York McGraw Hill

Baker B L (1942) *Anat Rec* 83 47

Bauer W and Aub J C (1941) *J clin Invest* 20 295

Blumenthal, H T and Loeb L (1942) *Endocrinology* 30 502

Campbell I L and Turner C W (1942) *Res Bull Mo agric Exp Sta* No 352 134

Castleman B and Hertz S (1939) *Arch Path* 27 69

Cushing H and Davidoff L M (1927) *Monogr Rockefeller Inst med Res* No 22

Engfeldt B (1950) *Acta endocr (Kbh)* suppl 6

Erdheim J (1903) *Beitr path Anat* 33 158

Greep R O (1948) *In* The Hormones Physiology Chemistry and Applications 1 255 eds Pincus G and Thimann K. V New York Academic Press

Ham A W and Haist R E (1939) *Nature (Lond)* 144 835

Harrison M and Fraser R (1959) *J Endocr* 18 25

Hertz S and Kranes A (1934) *Endocrinology* 18 350

Houssay B A (1936) *Harvey Lect* 31 116

Kellgren J H Ball, J and Tutton G K (1952) *Quart J Med* 21 405

Li C H and Papkoff H (1956) *Science* 124 1293

Moldawer M P Nardi G L and Raker J W (1954) *Amer J med Sci* 228 190

Panel appointed by the Clinical Endocrinology Committee of the Medical Research Council (1959) *Lancet* 1 7

Perlman R M (1944) *J clin Endocr* 4 483

Rupp J J and Paschkis K. E (1953) *Ann intern Med* 39 1103

Sheehan H L and Summers V K (1949) *Quart J Med* 18 319

Shelburne S A and McLaughlin C W (1945) *J clin Endocr* 5 232

Stoerk H C and Carnes W H (1945) *J Nutr* 29 43

Törnblom N (1949) *Acta Endocr (Kbh)* suppl 4

Underdahl, L O Woolner L B and Black B M (1953) *J clin Endocr* 13 20

Waine H Bennett B A and Bauer W (1945) *Amer J med Sci* 209, 671

DISCUSSION

Luft I was surprised to hear that the parathyroid glands were enlarged in acromegalic patients I suppose they were weighed I recall that for some time I was surprised to find what I thought were enlarged parathyroid glands in hypophysectomized patients at autopsy When we started to weigh the glands we found normal values which means that the parathyroids are normal in size after hypophysectomy We have not studied the histology of these glands

Russell Fraser The parathyroid glands of our acromegalic patient *showed at histology rather enlarged hypertrophied looking* cells I am not a pathologist but I understand from my colleagues that the assessment of the weight of the parathyroid gland as an index of parathyroid activity is an unreliable criterion especially when the effect of age is not taken into account With advancing age there is a steady increase in infiltration of fat in strands inside the parathyroid glands perhaps the most reliable criterion is that of cell diameter or criteria concerning the nucleus These may have to be looked at very carefully before the condition of a gland in hypopituitarism can be assessed I think the paper by Sheehan and Summers (1949 *loc cit*) is the most reliable one in favour of it they do indicate lowered activity and atrophy It is a replacement by fat rather than a shrinkage of the gland

Sonenberg It is known that thyroid hormone will induce calciuria either manifested by hyperthyroidism or after single injection I presume that your parathyroidectomized animals were also thyroidectomized If they were not it minimizes my point I still wonder about the role of the thyroid in inducing some of this calciuria

Russell Fraser We wondered about that too I believe that it may turn out that the way in which the calciuria is induced by the thyroid may have something in common with the way in which growth hormone induces it but the latter is not mediated via the thyroid The evidence for that is that some of these patients thyroid function was very carefully tested before and during the growth hormone injections and there was no evidence of a thyrotropic effect With regard to the rats as I have mentioned they did have thyroids of normal size and growth hormone was the only hormone injected

Raben Did the parathyroidectomized rats grow in response to growth hormone?

Russell Fraser All of the rats whether parathyroidectomized or not showed the growth effect

Li You report an effect of growth hormone on the calcium and strontium excretion in hypophysectomized rats have you found that in normal rats?

Russell Fraser Again it may be a question of dosage In the dose level used there (3 mg /day for three days) it did not produce any effect on the normal rat Therefore we went straight on to the experiment on hypophysectomized rats because even if we cannot explain it it is fairly usual that most animals are more sensitive to the hormone of a gland which they do not possess I suspect that the effect might be obtainable in normal animals with a larger dose With regard to normal animals I can only say that on human patients (of normal endocrine status) we have observed this calciuric effect but it was much smaller and of shorter duration

Li Some years ago Dr Ulrich found that the deposition of radioactive calcium on the uncalcified cartilage of the tibia in hypophysectomized rats was affected by beef growth hormone (Ulrich F Reinhardt W O and Li C H (1951) *Endocrinology* 49 213) The effect in hypophysectomized animals was quite striking compared with the normal controls We did not make any precise determinations of the urinary calcium or strontium but as far as I remember in the normal animal the level of urinary calcium is not affected by beef growth hormone in a dosage of as high as 5–10 mg daily I am sure that Prof Young will agree with me that even 3 mg /day of growth hormone is a very high dose in hypophysectomized rats

Beck Prof Russell Fraser what was the dietary phosphate in your rats low or high?

Russell Fraser It was planned to be of normal intake level

Beck I have been interested in these observations which Prof Russell Fraser showed me recently in London I have been searching the literature for an explanation of them and also of our findings in human beings I found a very interesting experiment reported by Copp some years ago (Copp D H (1955) *In* The Hypophyseal Growth Hormone Nature and Actions p 186 eds Smith R W Jr Gaebler O H and Long C N H New York McGraw Hill) He was able to show that if the phosphate intake of rats receiving growth hormone was decreased then one got a marked mobilization of skeletal minerals so that the urinary calcium rose and there was a marked demonstrable demineralization of the skeleton This might be another explanation for the observations that you have made If the phosphate intake was normal this influence did not occur and he concluded that the primary effect of growth hormone on bone was on the deposition of the protein matrix and that mineralization came later and that if the demands for phosphate for protein

deposition were higher than the phosphate intake then there was demineralization to supply this phosphate

Russell Fraser I am familiar with this work and I am sure that the effect may be observed, but I do not think that this has any connexion with our observations In chronic replacement dosage you must have phosphate there since the major effect of growth hormone may be on the formation of new bone and on the growth of new bone There is no question that growth hormone makes bone grow Here we are observing an additional effect which is probably mediated by some such thing as the citrate effect which we have already seen which may affect the ionized calcium level in the cell and will only do so when high enough doses of growth hormone are given In these patients we have measured the calcium uptake in bones by tracer tests and there is no significant effect on the uptake of the tracer in these short experiments It is a condition more comparable to the status in calcium deficiency or when ionized calcium is pushed down artificially by some other metabolic means This is a subsidiary effect and not the main effect of growth hormone on bone It may be analogous to the effect of calcium deficiency itself in that condition you get exactly the same picture in the bones i e osteoporosis not osteomalacia and stimulated parathyroid glands and if you remove the hypophysis you do not get parathyroid stimulation

Loraine What is your opinion regarding the possibility of assaying parathyroid hormone in body fluids ? If this were feasible the results obtained might well strengthen the hypothesis which you have put forward

Russell Fraser Dr Davis assay requires 40–50 mice it is perfectly possible to demonstrate the difference between the urine of a patient with parathyroid tumour and normal urine (Davis B (1958) *J Endocr* 16 369) We no longer do that assay because it constitutes a very expensive one to us It would be a very satisfactory way of proving that this is a parathormone action but I would submit that it is not very easy to explain our results in any other way unless perhaps for some peculiar reason the metabolic effect of growth hormone on bone is only possible with a normal supply of parathormone

Luft Prof Russell Fraser you mentioned citrate this seems to be of great significance in bone metabolism as has been so nicely shown by Neuman If you could increase in some way the citric acid concentration around the bone cells the solubility of calcium would also be increased and you might get a movement of calcium from bone to extracellular fluid and to urine We have no data about growth hormone and the metabolism of citrate except that an extra

increase in citrate in the blood of two patients was observed during glucose infusion when growth hormone was also given From your paper I could not understand where the parathyroids come in May I also take the opportunity in this connexion to mention that Drs D Alexandrides and D Ikkos in my Department have been measuring the disappearance rate of ^{85}Sr in different groups of patients especially with bone disease and with acromegaly The only group that showed some change from normal was the acromegalic one in that one there was a significant decrease in the biological half life of ^{85}Sr

Russell Fraser That was our first hypothesis too i e that it was a direct citrate effect on bone But I believe that the great importance of having parathyroids to get the effect make it more likely that the citrate which is probably changed much more in the cells than it is in the serum may be influencing more prominently the secretion of parathormone than acting directly on the bone It is possible that the explanation could arise from the citrate only influencing normal bone in the presence of parathormone in normal supply But this is not the experience of people who infused citrate It has been possible to infuse citrate into the abdomen and get quite a large release of calcium from parathyroidectomized animals though perhaps not at the same blood level as when the animal has parathyroids We are in the middle of assaying citrate in various tissues and are hoping to understand the mechanism

Luft Do I understand correctly that you consider that the parathyroid gland has to be present in order to get this effect ?

Russell Fraser Yes

Pearson Prof Russell Fraser have you made any studies on prolonged administration of growth hormone and have you obtained the same effects ? In our experience with prolonged administration of growth hormone to hypophysectomized adult patients the change in urinary calcium was very sporadic In fact it was very infrequent with doses of 5–10 mg /day It is much more common in the pituitary dwarfs In one patient there was hypercalciuria initially but after continuous administration of growth hormone for a 14 to 21 day period the hypercalciuria subsided even though we continued to get marked anabolic effects

Russell Fraser We have not had much experience with prolonged administration of growth hormone We have given growth hormone about 2–5 mg twice a week over periods of three months With these dose levels we do not observe this calciuric effect or at least it is very minimal Therefore we cannot say how long it would last with dose levels large enough to produce it

Pearson Do you conclude that the osteoporosis of acromegaly is due to secondary hyperparathyroidism ?

Russell Fraser I believe that all osteoporosis could be due to excessive resorption and not due to defective deposition of bone Whether you have osteoporosis or osteitis fibrosa cystica may be a matter of degree Slightly negative calcium balance usually produced by excessive resorption of bone may lead to osteoporosis and if more severe to osteitis fibrosa This is very well illustrated with the thyroid hyperthyroidism more commonly produces osteoporosis and occasionally patchy osteitis fibrosa also hyperparathyroidism in milder degrees may produce no bone lesion the first bone lesion would be osteoporosis Acromegaly I agree with you may be attributable to excessive bone resorption mediated by the parathyroid

Li Do you consider that your observed effect is a direct one of growth hormone ? In other words is it one of the intrinsic properties of growth hormone to act directly on the parathyroids ? You showed clearly that in parathyroidectomized rats growth hormone has no effect on calcium or strontium excretion We found in many cases an unexpected intrinsic activity of growth hormone in other organs of the rat not only on body weight growth but also on the sum effect on adrenals ovaries and ventral prostate Perhaps the effect that you observe is that the growth hormone acts directly on the production of parathormone which in turn increases the citric acid level You may recall the hypothesis proposed by Dr Neuman and his co workers from Rochester i e that the parathyroid function is the production of the citric acid level in blood so that the solubility of calcium increases (Neuman W F *et al* (1958) *J Amer chem Soc* 80 620) Do you consider that as a possibility ?

Russell Fraser In my view it is probable that it is an intracellular effect of growth hormone Since one obtains the effect without alteration in the serum level of calcium which implies that there is a little excess citrate in the serum it seems to me unlikely that it should be only occurring in the parathyroid glands

The effect of parathormone on bone may also be somewhat enhanced by the excess of citrate I should think that all these three effects are attributable to growth hormone That may be one of the reasons why hyperparathyroidism along with growth hormone excess is *clinically different from that of simple hyperparathyroidism*

de Ulhoa Cintra Would you not expect a change in phosphate excretion in these patients at a level comparable with the amount of calcium excretion in the urine ?

Russell Fraser As I mentioned we calculated the expected combined effects on phosphate excretion from both our calcium effect on the one hand (which would increase the phosphaturia) and also from our nitrogen retention effect on the other hand which would

decrease the phosphaturia We did these calculations day by day for each individual patient Thus we obtained a line almost parallel with the observed phosphorus excretion Therefore we drew the conclusion that the observed phosphate effects in these acute growth hormone experiments were entirely indirect and completely attributable to either the anabolic or the bone dissolving effect

Pearson But there was no excess phosphate excretion as you would expect in increased secretion of parathormone

Russell Fraser Yes but there was a far greater nitrogen anabolism laying down the muscle tissue I think that you would agree that this takes quite a bit of phosphorus with it

Pearson Did you make a correction for nitrogen and calcium and find more phosphate than you could account for by positive nitrogen and calcium balance?

Russell Fraser That is what we did We calculated the theoretical phosphorus excretion using the primary data of our calcium and nitrogen balances thus ascertaining whether we could expect an increase of phosphorus excretion because of predominant bone dissolving effect or a decrease because of predominant anabolic effect on protoplasm The results obtained day by day gave us a theoretical phosphorus excretion extraordinarily close to the observed value

Pearson Is it not a characteristic effect of the parathormone to cause initially phosphate diuresis which cannot be accounted for on the basis of nitrogen and calcium?

Russell Fraser That is a possibility that might have to be considered but we must remember that when parathormone is given alone it may have an effect on the renal clearance very different from the effect obtained when growth hormone is given as well It is a striking feature of the growth hormone effect that we have a great calcium excretion but no change in serum calcium That is also what we saw with phosphate It seems that the urinary excretion does not have very much relationship to its blood level

de Ulhoa Cintra Have you established in your hyperparathyroid patients the amount of calcium in urine as compared with the level of serum calcium? Is it greater in those cases as compared with the ordinary parathyroid patients?

Russell Fraser The more one studies hyperparathyroid patients the less easy it is to understand the relationship between serum calcium and urinary calcium We have seen patients who had absolutely normal levels of urinary calcium with serum calcium in the region of 15–16 mg but we have seen other patients with hyperparathyroidism with their urinary levels vastly in excess of the normal and a slightly raised serum level I cannot explain these

differences, but I suspect that the associated responses in other endocrines may be much more complicated in hyperparathyroidism than we think At least the clearance does not stay the same in all patients

Luft Concerning the question about bone disease in acromegaly has it been proved to be osteoporosis?

Russell Fraser What would constitute proof? Osteoporosis constitutes thin bones without explanation!

Luft Do you think that acromegalics have thin bones'?

Russell Fraser Yes I think so A collapse of the spine occurs with the kyphosis and an abnormal translucency It is very difficult to say it is proved I agree but they do have the metabolic osteoporosis if you won t have the rest—increased urinary calcium excretion negative calcium balance That has often been shown in acromegaly

de Ulhoa Cintra We have done some studies on urinary excretion of mucoproteins in hyperparathyroid patients and found it be very high three to four times that in normal subjects We have hyper parathyroids with weak bones Perhaps this would help in trying to see if the hyperparathyroids are already hyperactive in this condition

de la Balze Prof Russell Fraser, would you comment on the direct action of growth hormone on the renal clearance of calcium?

Russell Fraser It is necessary to study the fractionation of cal cium in the urine and in the serum further than we have yet done before we can interpret the finding of an increased calcium excretion without a change in the blood level It could be an alteration in renal function It could equally be an alteration in the mode in which calcium is carried in the serum calcium citrate being vastly more readily cleared I favour that hypothesis

Gómez Mont The effects that you report can be interpreted as being the result of hypocalcaemia secondary to parathyroidectomy Have you measured the serum calcium in the hypophysectomized animals after the removal of the parathyroid glands?

Russell Fraser It is a possible hypothesis I agree It could be the case that you only get this effect in the presence of normal para thyroid secretion and that we are not stimulating the parathyroid though I think it is less likely

Wilhelmi Prof Russell Fraser had you thought of beginning to sort out some of the growth hormone effects on serum calcium by studying nephrectomized animals? On the basis of your tentative hypothesis that there may be some calcium binding substance pro duced in response to growth hormone nephrectomy might still not inhibit the very marked rise in serum calcium that one could expect after administering the hormone

Russell Fraser We have thought of this Prof Wilhelmi To do nephrectomy would take us into a difficult field and it is particularly complicated in this respect by the known tendency of serum citrate to rise very strikingly with nephrectomy or uraemia from any cause This would make it very difficult to interpret our effects

Wilhelmi Some data have been published indicating marked renal effects of human growth hormone in humans (Gershberg H and Heinemann H O (1957) *J clin Endocr* 17 377 Gershberg H personal communication) and I wonder to what extent these effects on the kidney might in turn be reflected on the parathyroid and play a part in the phenomenon that you have observed If there are renal effects of this sort which may be playing a part then perhaps the most direct way of side stepping them would be to remove the kidneys I agree with you that it invites one into a very messy situation you will recall the great controversy that raged over the question of whether the parathyroid hormone has an effect on bone In the course of using nephrectomized animals people got as many different answers as there were experiments

Russell Fraser One of my colleagues tried the effect of growth hormone on a patient with one of these phosphate leak renal lesions which produce rickets to see whether it was possible to influence this type of lesion by the direct action of growth hormone The results are preliminary but it seems that the response to the growth hormone may be different in the first place and in the second place it does not alter the phosphate leak

THE VARIABILITY IN PHYSIOLOGICAL RESPONSE TO GROWTH HORMONE*

J C BECK E E McGARRY I DYRENFURTH
R O MORGEN E BIRD AND E H VENNING

McGill University Clinic Royal Victoria Hospital Montreal

Introduction

EXTENSIVE reviews on the biochemical nature of growth hormone and the multiplicity of its physiological actions have been made by a number of investigators in an international symposium (Smith Gaebler and Long 1955) by Astwood (1955) Randle (1957) Ketterer Randle and Young (1957) Li (1956) Hays and Steelman (1955), and most recently by Raben (1959) Our initial observations on the metabolic effects of primate growth hormone in man (Beck *et al* 1957) have been amply confirmed by many investigators using human growth hormone (HGH)† prepared by a variety of techniques (Henneman 1959 Henneman *et al* 1958, Pearson *et al* 1957 Bergenstal 1959 Werner and Bartter 1958 Crigler Knopp and Chagnon 1958 Ikkos Luft and Gemzell 1958 Luft *et al* 1958 Medical Research Council 1959) Since this initial report our observations have been extended to include metabolic balance studies in twelve patients and a more extensive review of our experiences in the first eight studies including the experimental methods used is reported elsewhere (Beck *et al* 1960) These studies were carried out in seven subjects three with dwarfism one who had been

* Supported in part by grants from The National Research Council of Canada The National Cancer Institute of Canada and The United States Public Health Service Grant Number 289–60

† We wish to acknowledge the assistance of Dr M Raben in the preparation of human growth hormone used in the earlier studies and Nordic Biochemicals Ltd Montreal for their aid in the collection and extraction of the growth hormone used in subsequent studies

hypophysectomized because of metastatic carcinoma of the breast one with postmenopausal osteoporosis a normal medical student and a patient with chronic renal disease One pituitary dwarf was studied on two occasions while the hypophysectomized patient was studied first only during a few days and subsequently during a more prolonged period on HGH

Results

Results are presented in an abbreviated fashion to highlight the description of the variability in physiological response to growth hormone, and are best discussed under the headings of the particular parameter of growth hormone activity studied (Figs 1–4)

A significant fall in blood urea nitrogen occurred on the second third or fourth day of HGH administration in the four patients in which this observation was made The patient with chronic renal disease failed to demonstrate an unequivocal fall until the twentieth day after initiation of growth hormone and this became maximal on the twenty seventh day The decrease usually persisted for two or three days after cessation of growth hormone administration Alterations in the serum non protein nitrogen (NPN) have been recorded in five studies and became apparent between the second and sixth day of HGH administration In the patient with renal disease the fall persisted for 15 days after discontinuance of growth hormone whereas it usually returned to control values in 2–3 days Spontaneous day to day varia tions or an insufficient number of determinations made the interpretation of the changes in the serum non protein nitrogen more difficult in the three remaining studies

Prompt nitrogen retention as measured by metabolic balance techniques is usually apparent on the second day of HGH administration maximal on the third to the sixth day of treatment and may persist for 2–3 days after discontinuance of growth hormone In the patient with chronic renal disease the effect persisted for at least 18 days Positive nitrogen

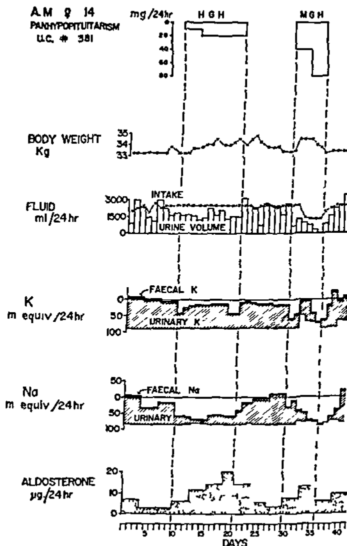


Fig 1

Figs 1–4 Human growth hormone was administered for 11 days monkey growth hormone for 6 days to a 14-year old male with well documented hypopituitarism secondary to a craniopharyngioma In these and the subsequent metabolic balance charts the intake is plotted from the zero line downward and the urinary excretion is plotted upward from the intake line above which is the faecal excretion. A negative balance is represented by cross hatched areas above the zero line while a positive balance is represented by a clear area below the line

balance was demonstrated in six of the eight studies while in two patients no change was observed One of these, a patient

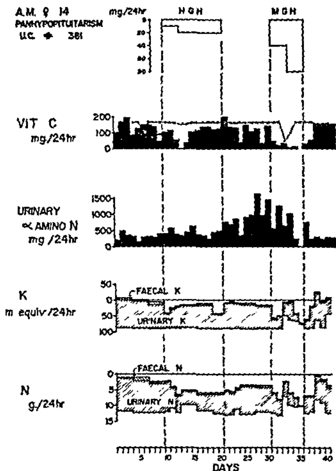


FIG 2

with postmenopausal osteoporosis, had shown a previous response to a variety of androgenic steroids In two patients a decrease in nitrogen intake during the period of HGH

administration resulted in a reduction in the degree of nitrogen storage

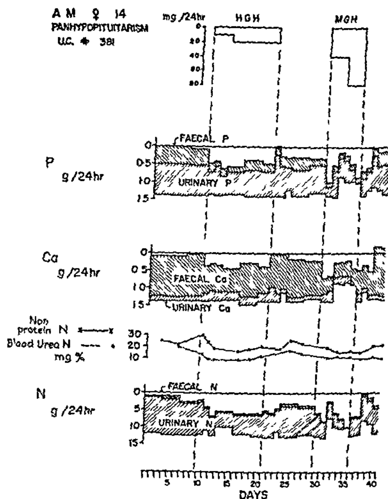


Fig 4

The retention of potassium during HGH administration closely paralleled that of nitrogen in three patients De creased potassium excretion usually occurred on the first day

of HGH administration and increased excretion within 2 days after discontinuance of HGH Monkey growth hormone (MGH) produced marked potassium storage in the one individual to whom it was given In the other subjects, there was little change in the potassium balance when the period of

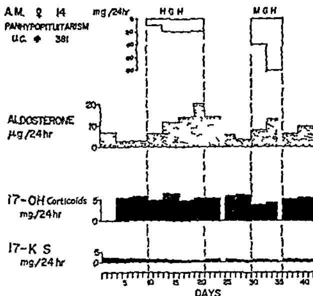


FIG 4

HGH injection was compared with the pre growth hormone control period All patients showed potassium loss in the post growth hormone control period but this could not convincingly be considered a ' rebound in potassium excretion

Four subjects showed an increase in urinary calcium excretion while receiving HGH This was evident on the first day maximal on the fourth to seventh day and decreased promptly on the discontinuance of growth hormone The positive calcium balance was usually achieved by a fall in

faecal calcium, despite the previously described rise in urinary calcium In three patients calcium retention was demonstrable during growth hormone administration while in others it occurred more slowly and became maximal after cessation of growth hormone This "delayed response" thus persisted beyond the time of return to nitrogen equilibrium

Phosphorus retention was observed in five subjects, while the remainder showed no consistent change in urinary or faecal phosphorus excretion in association with HGH administration In four subjects the phosphorus retention was achieved by decreased excretion in both the urine and the faeces The same feature of a prolonged period of phosphate retention in the post growth hormone control period previously described for calcium, was observed ("delayed response")

A positive sodium balance was observed in five subjects during HGH administration The decrease in urinary sodium did not persist long after withdrawal of growth hormone and a suggestion of a "rebound" in urinary sodium excretion was seen, although the amount of sodium lost was less than the cumulative positive sodium balance

A significant rise in urinary aldosterone excretion during HGH administration was seen in five patients, being most marked with a growth hormone dose of 15 mg per day or more One patient showed no change in aldosterone excretion while in another interpretation of the data is difficult The positive sodium balance coincided with the increase in urinary aldosterone output in three patients but this may be purely coincidental in that two patients with a marked increase in urinary aldosterone showed insignificant changes in sodium balance

The failure to demonstrate any alteration in urinary 17 ketosteroid and 17 hydroxycorticosteroid excretion during HGH administration suggests that adrenocorticotropic hormone (ACTH) contamination in the Raben preparation is minimal or absent Absence of a significant change in ^{131}I uptake and urine osmolality during HGH administration is strong supportive evidence for the absence of thyroid

stimulating hormone (TSH) and antidiuretic hormone contamination respectively

A fall in urinary ascorbic acid during HGH administration was seen in three subjects while in another three only equivocal changes were observed Alterations in urinary α amino nitrogen have only been seen in severe hypopituitary subjects and interpretation of these data must await further study

No significant change in the serum concentration of sodium potassium chloride bicarbonate calcium cholesterol cholesterol esters total lipids lipid phosphorus mucoproteins or

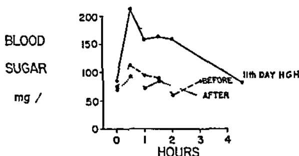


FIG 5 Impairment of the oral glucose tolerance curve during human growth hormone administration in a dose of 10 mg per day for 3 days increased to 20 mg per day for the subsequent 8 days

glucosamine has been observed during HGH administration Although the serum inorganic phosphorus showed a tendency to rise during short term growth hormone administration sustained elevations occurred only during prolonged treatment This was also true for the serum alkaline phosphatase

Human growth hormone in a dose ranging from 200 to 600 μg /Kg /day in hypopituitary subjects on cortisone replacement therapy has consistently led to impairment of carbohydrate tolerance (Fig 5) This has been manifested by an elevation in the fasting blood sugar an increase and occasional delay in the peak blood sugar level and a marked elevation in the two hour post cibal blood sugar level using

the oral or a modified oral glucose tolerance test These patients have all shown flat glucose tolerance curves after discontinuance of growth hormone In the fasting subject we have observed hypoglycaemia to occur within 30 minutes of a single intramuscular injection of HGH

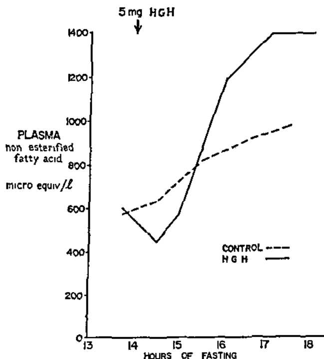


FIG 6 The effect of human growth hormone on the plasma non esterified fatty acids in the fasting normal subject

Evidence for the fat mobilizing properties of HGH has been obtained by observing its influence on the plasma levels of non esterified fatty acids in the fasting human subject Following a single intramuscular injection of 5 mg of growth hormone there is an initial fall in plasma non esterified fatty acids which is maximal between 15 and 45 minutes followed by a rise which in the endocrinologically intact individual is maximal between 4 and 8 hours (Fig 6) There is little if any

difference in the non esterified fatty acid response of normals and patients with hypopituitarism using this dose of growth hormone and following the response for 8 hours The non esterified fatty acid response appears, in our hands to be the earliest demonstrable activity produced by growth hormone in the fasting subject whereas a fall in blood urea nitrogen or serum non protein nitrogen is seen first in the non fasting individual

As this data has already intimated anabolic effects have been demonstrated in most but not all of the individuals studied One subject with dwarfism and another with severe postmenopausal osteoporosis failed to reveal a retention of the major components of protoplasm although other physiological activity was present Further dissimilarity in physiological response to growth hormone has been observed and these are best dealt under the following headings

(1) the anabolic effect of growth hormone and its influence on carbohydrate metabolism
(2) the anabolic effect of growth hormone and its influence on aldosterone excretion
(3) variations in the early response of blood glucose and plasma non esterified fatty acid levels with different batches of HGH

(1) The anabolic effect of growth hormone and its influence on carbohydrate metabolism

Our data confirm the well established observations in other species that growth hormone may be diabetogenic The diabetogenic effect could not always be related to the anabolic effect of the hormone as judged by nitrogen retention since impairment of glucose tolerance occurred with no evidence of anabolic activity This was observed in a dwarf in whom the maximal dose of human growth hormone was 440 µg /Kg /day (Figs 7 and 8) In addition impaired glucose tolerance occurred in the absence of nitrogen retention when a modified porcine growth hormone prepared by Raben was given to two subjects with dwarfism In two individuals one a normal

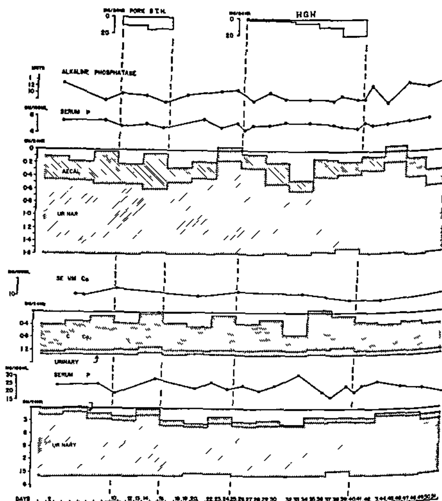


Fig 7 Metabolic balance study in an 18 year old dwarf receiving a modified porcine growth hormone preparation followed by human growth hormone Note the absence of any significant change in nitrogen calcium and phosphorus balance

Pork S T H = modified porcine growth hormone prepared by Raben

student the other a patient with chronic renal disease no change in glucose tolerance was seen although unequivocal nitrogen retention occurred In addition no alteration resulted from the administration of large doses of MGH in the

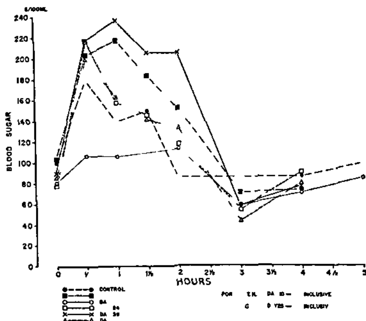


FIG 8 Oral glucose tolerance curves in the patient depicted in Fig 7 Note the impairment of carbohydrate tolerance on both porcine and human growth hormone

Pork S T H = modified porcine growth hormone prepared by Raben.

one pituitary dwarf to whom it was given although it produced the most marked anabolic response which we have seen (Fig 9) Finally a patient with postmenopausal osteoporosis and a diabetic type of curve in the control period showed a normal curve during growth hormone treatment

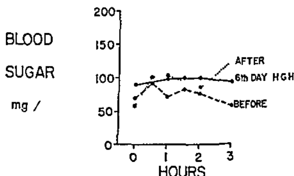


Fig 9 The failure of monkey growth hormone to alter the oral glucose tolerance curve in a pituitary dwarf (Dose 40 mg per day for 3 days followed by 80 mg per day for the subsequent 3 day period) This is the patient depicted in Fig 5

Fig 10 The response of the urinary aldosterone as measured by both chemical assay and bioassay in a dwarf receiving human growth hormone This is the patient depicted in Fig 5 in whom no anabolic effect of growth hormone could be demonstrated

Pork S T H = modified porcine growth hormone prepared by Raben

(2) The anabolic effect of growth hormone and its influence on aldosterone excretion

In four of the studies an increase in urinary aldosterone could be correlated with nitrogen retention while in two patients

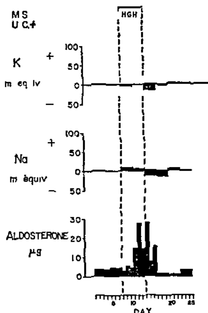


FIG 11 The influence of human growth hormone on urinary aldosterone excretion in a patient with postmenopausal osteoporosis A dose of 5 mg per day was administered for 3 days followed by 15 mg per day for the succeeding 3 days and this failed to result in the retention of any of the protoplasmic constituents

(dwarfism and postmenopausal osteoporosis respectively) no alteration in nitrogen storage occurred under circumstances in which a definite increase in urinary aldosterone could be demonstrated (Figs 10 and 11) In the normal student nitrogen retention occurred without any alteration in urinary aldosterone excretion

(3) Variations in the early response of blood glucose and plasma non-esterified fatty acid levels with different batches of growth hormone

A fall in blood sugar and in plasma non esterified fatty acid levels is usually seen within the first 15–45 minutes after the intramuscular injection of 5 mg of HGH into the fasting human subject In a series of studies using a different batch of growth hormone the transient fall in these parameters failed to occur although the subsequent rise in plasma non esterified fatty acid levels was unchanged

Discussion

The data concerning the metabolic effects of primate growth hormone in man are as yet relatively meagre and although they permit some firm conclusions to be drawn interpretation of other observations must of necessity be tentative This is particularly true of certain dissimilarities in growth hormone activity which have been presented not because an explanation is available but in the hope that a discussion might delineate further methods of clarifying these issues

A number of hypotheses can be proposed as explanations for the apparent dichotomy between the anabolic and diabetogenic effects of HGH It would seem most unlikely that the growth and diabetogenic principles were not identical in the light of the great increase in knowledge concerning the molecular structure of growth hormone

In the experimental animal a variety of factors influencing the growth and diabetogenic responses have been observed among them age and species of animal pregnancy and lactation the availability of thyroxine adrenocortical steroids and dietary composition In the dog the ease of development of diabetes during growth hormone administration has been shown to be related to the amount of protein in the diet dogs receiving a high carbohydrate or a high fat diet were less sensitive to the diabetogenic action of growth hormone than dogs fed a high protein diet (Young 1949) In our observa

tions none of these factors seem to play a rôle The protein intake in our subjects varied from 0 81 to 2 92 g /Kg /day, but this variation could not be related to the development of the impaired carbohydrate tolerance

There seems to be no doubt that the dose of growth hormone is an important factor in the manifestation of its diabetogenicity Raben (1959) has suggested that doses of the order of 30–100 µg /Kg /day result in ketosis and hyperglycaemia only in the hypophysectomized diabetic subject The changes which have been described in our studies occurred with doses ranging from 200–600 µg /Kg /day In addition in the more prolonged studies performed to date by Raben Henneman and ourselves no impairment of glucose metabolism has occurred with intermittent therapy The amount of growth hormone administered however does not readily explain our findings

It is of interest that the two individuals in whom anabolism occurred without any real effect on carbohydrate tolerance were endocrinologically intact subjects (a normal student and a patient with chronic renal failure) in contrast to the group with hypopituitarism These observations are in agreement with those reported in the experimental animal where diabetogenicity of growth hormone is most readily demonstrable in the absence of pituitary function This however does not explain the impairment of carbohydrate tolerance in the absence of an anabolic effect or the apparent failure of monkey growth hormone to be diabetogenic

The effect of HGH in increasing urinary aldosterone is of interest in relation to the as yet incompletely defined efferent pathway controlling its secretion Our observations suggest that the increase is dependent in part upon the amount of growth hormone administered The possibility that the present preparations of primate growth hormone also contain a contaminant capable of stimulating aldosterone secretion has been considered A possible alteration in the metabolic degradation of aldosterone increasing the free fraction as measured in this study without altering the total aldosterone excretion might also explain these findings but preliminary

data suggest this to be incorrect Another possibility is that growth hormone acts by stimulating a humoral agent concerned with aldosterone secretion or that it undergoes an *in vivo* alteration this issue is partially clarified by Dr Venning (this colloquium p 174)

The concomitant fall in blood glucose and plasma non esterified fatty acid levels after HGH administration to the fasting subject certainly suggests an insulin like action Our data, at present do not help in determining whether this is due to insulin release from the pancreas or the release of insulin bound to tissues or to the plasma proteins or whether it is completely unassociated with insulin activity The absence of this effect with a certain batch of growth hormone might suggest that minor alterations in the molecule may lead to an alteration in physiological effect a concept which has a parallel in the behaviour of corticotropin

Summary

There seems to be unanimity among the many investigators who have utilized human growth hormone that it is physiologically active in the human and appears to reproduce in man many of the effects that have been previously described in a variety of experimental animals Anabolic effects have been observed in most, but not all of the individuals studied Certain dissimilarities in physiological response to growth hormone have been observed between its anabolic effect and its influences on carbohydrate metabolism as well as its anabolic effect and its influences on aldosterone excretion Alterations in blood glucose and plasma non esterified fatty acid levels appear to vary with different batches of human growth hormone Certain hypotheses as an explanation for these observations are discussed

REFERENCES

Astwood E B (1955) *In* The Hormones Physiology Chemistry and Applications 3 235 eds Pincus G and Thimann K V New York *Academic Press*

BECK J C McGARRY E E DYRENFURTH I and VENNING E H (1957) *Science* 125 884
BECK J C McGARRY E E DYRENFURTH I VENNING E H BIRD E and MORGEN R O (1960) *Metabolism* in press
BERGENSTAL D (1959) Personal communication
CRIGLER J F KNAPP J A and CHAGNON J (1958) *A M A Amer J Dis Child* 96 432
HAYS E E and STEELMAN S L (1955) *In* The Hormones Physiology Chemistry and Applications 3 201 eds Pincus G and Thimann K V New York Academic Press
HENNEMAN P H (1959) Personal communication
HENNEMAN P H MOLDAWER M COWELL E L MITCHELL M and WADDELL M J (1958) Endocr Soc Meeting San Francisco abstr 40
IKKOS D LUFT R and GEMZELL C A (1958) *Lancet* i 720
KETTERER B RANDLE P G and YOUNG G F (1957) *Ergebn Physiol* 49 127
LI C H (1956) *Advanc Protein Chem* 11 101
LUFT R IKKOS D GEMZELL C A and OLIVECRONA H (1958) *Lancet* i 721
Panel appointed by the Clinical Endocrinology Committee of the Medical Research Council (1959) *Lancet* i 7
PEARSON O H LIPSETT M B GREENBERG E and ROY B S (1957) Endocr Soc Meeting New York abstr 40
RABEN M S (1959) *Recent Progr Hormone Res* 15 71
RANDLE P J (1957) *Symp Soc exp Biol* 11 183
SMITH R W Jr GAEBLER O H and LONG C N H eds (1955) The Hypophyseal Growth Hormone Nature and Actions New York McGraw Hill
WERNER I and BARTTER F C (1958) Endocr Soc Meeting San Francisco abstr 41
YOUNG F G (1949) *Proc roy Soc Med* 42 321

[Discussion of this paper was postponed until after the short communication by Drs Venning and Lucis —Eds]

Short Communication

GROWTH HORMONE AND ALDOSTERONE SECRETION

ELEANOR H VENNING AND O J LUCIS

McGill University Clinic Royal Victoria Hospital and Department of Investigative Medicine McGill University Montreal

THE observations made in our department that monkey and human growth hormone when administered in amounts above 5 mg daily to patients with pituitary insufficiency caused a rise in the levels of urinary aldosterone have prompted us to study the mechanism of action of this hormone on aldosterone secretion

Various preparations of growth hormone derived from human, monkey and porcine tissue were kindly supplied by Dr Raben Dr Wilhelmi and by Mr Antoff of Nordic Biochemicals

The first experiments were designed to determine whether growth hormone had a direct effect on the isolated adrenal gland Tissue from rat and human adrenal glands was incubated in Krebs Ringer glucose solution and varying amounts of the different preparations of growth hormone were added to the incubation mixture The secretion rates of aldosterone and other corticosteroids were measured and expressed as µg /g /hour

When human, porcine or monkey growth hormone was added to the incubation medium of rat adrenal tissue in varying amounts, none of these preparations had any significant effect on the rate of secretion of aldosterone and corticosterone (Table I)

Similar studies were carried out on human adrenal tissue obtained from two patients with Cushing s syndrome The

Table I

EFFECT OF GROWTH HORMONE ON ISOLATED RAT ADRENAL GLAND

Growth hormone mg /g tissue	*Aldosterone* µg /g tissue/hour	*Corticosterone* µg /g tissue/hour
I Human (Raben)		
none	10 1	16 4
0 181	10 9	15 3
0 587	9 0	15 8
1 085	9 0	lost
II Porcine—205 A (Wilhelmi)		
none	11 8	21 8
0 042	9 9	20 5
0 167	12 8	22 6
0 418	11 2	23 9
III Monkey—462 B (Wilhelmi)		
none	12 8	25 0
0 039	13 9	27 1
0 154	11 7	25 0
0 368	12 6	26 0

rates of secretion of aldosterone hydrocortisone cortisone corticosterone and 11β hydroxy Δ^4 androstenedione were measured (Table II)

In Case 1 0 15 mg /g tissue human growth hormone prepared by Dr Raben had no effect on the secretion rate of any of the five steroids With larger amounts 1 5 mg /g tissue all the steroids were increased and this was considered to be due possibly to contamination of the preparation with small amounts of adrenocorticotropic hormone (ACTH) In the second case the human growth hormone was prepared by Nordic Biochemicals according to the procedure of Dr Raben Incubation time was increased to 6 hours and the medium was changed every 2 hours with a fresh amount of growth hormone added This preparation of growth hormone also caused an increased secretion of all these various steroids again suggesting the presence of small amounts of ACTH in the preparation

Adrenal tissue was also obtained from a patient showing a virilizing syndrome (Table III) Porcine and monkey growth hormone prepared by Dr Wilhelmi were used During the

Short Communication

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Table III

EFFECT OF GROWTH HORMONE ON ISOLATED HUMAN ADRENAL TISSUE (VIRILIZING SYNDROME)

	Growth hormone	*Aldosterone*	*Hydro-cortisone*	*Cortisone*	*Cortico sterone*	*11β OH Δ⁴ Androstenedione*
	mg /g tissue	μg /g tissue/hour				
Control (1–3 hours)	none	0 18	6 2	0 9	2 7	3 3
Porcine—*138F (Wilhelmi)*	0 36	0 21	13 6	2 1	4 5	5 1
Monkey—334B41 (Wilhelmi)	0 37	0 22	13 3	1 9	4 1	3 7
Control (3–5 hours)	*none*	0 14	4 7	0 6	3 4	3 3
Porcine—138E	1 70	0 29	15 5	2 3	6 8	5 7
Monkey—334B41	1 85	0 30	15 5	2 3	7 9	5 2

Table II

EFFECT OF GROWTH HORMONE ON ISOLATED HUMAN ADRENAL TISSUE (CUSHING S SYNDROME)

	Growth hormone	*Aldosterone*	*Hydrocortisone*	*Cortisone*	*Corticosterone*	11β *OH* Δ⁴ *Androstenedione*
	mg /g tissue	µg /g tissue/hour				
CASE 1	Human (Raben)					
	none	0 44	9 2	2 0	2 2	3 7
	0 15	0 49	9 0	1 9	2 0	3 4
	1 0	0 58	11 7	3 1	3 0	5 5
	Incubation time 2 hours					
CASE 2	Human—0357 HS (Nordic Biochemicals)					
	none	0 81	22 4	5 4	7 6	7 1
	0 69	0 98	32 8	6 5	13 4	7 7
	Incubation time 6 hours medium changed every 2 hours and fresh growth hormone added					

animals without an endogenous release of ACTH hypophysectomized rats were used to study the effect of growth hormone Two groups of hypophysectomized rats were used Group A were hooded male rats Twenty four hours after hypophysectomy 0 5 mg of monkey growth hormone (Wilhelmi 475–6C) was administered daily for 3 days Two hours after the last injection the adrenal glands were removed and incubated for 3 hours in Krebs Ringer solution The secretion rate of aldosterone was greater in the adrenals of the rats treated with growth hormone than in the adrenals of the control hypophysectomized rats while that of corticosterone was slightly lower (Table V)

A similar experiment was carried out in a second group of rats (Charles River strain) Group B which had been hypophysectomized for 11 days In these animals treatment with monkey growth hormone caused a still greater rise in aldosterone secretion with no effect on corticosterone secretion

In order to determine whether the plasma of the rats treated with growth hormone contained a tropic factor which would stimulate aldosterone secretion adrenals of normal rats were incubated in the plasma obtained from both groups of hypophysectomized rats and from the intact rats treated with growth hormone The results are shown in Table VI The rate of aldosterone secretion was higher in the adrenals incubated in plasma obtained from the rats treated with growth hormone than in the controls, while corticosterone secretion was unaffected in the first two experiments and was slightly increased in the third

Conclusions

These studies suggest that growth hormone does not directly stimulate aldosterone secretion *in vitro* When it is administered to intact and to hypophysectomized rats the adrenals of these animals secrete increased amounts of aldosterone The plasma of hypophysectomized rats treated with growth hormone appears to contain a substance which stimulates aldosterone secretion *in vitro*

incubation period 1–3 hours, 0 36 mg growth hormone per g of tissue was used At the end of this period the medium was changed and the tissue was incubated for an additional 9 hours with larger amounts of growth hormone In both incubation periods, these preparations caused a significant increase in the secretion of hydrocortisone, suggesting contamination of the preparation with ACTH Although the rate of aldosterone did increase this effect could not be dissociated from an ACTH effect and it had to be concluded that growth hormone did not appear to have a selective stimulating action on aldosterone secretion

Table IV

EFFECT OF TREATMENT WITH GROWTH HORMONE ON ADRENAL SECRETION OF STEROIDS

Male rats 180 ± 5 g received 2 injections daily of 0 9 mg of growth hormone Adrenals removed 2 hours after last injection and incubated in Krebs Ringer solution from 0–1 hour

	Aldosterone	*Corticosterone*
Porcine—205A (Wilhelmi)	µg /g /hour	µg /g /hour
Control rats	13 2	65 0
Growth hormone treated rats	15 3	66 0
Monkey—462B (Wilhelmi)		
Control rats	12 4	62 1
Growth hormone treated rats	16 2	78 6

The *in vivo* effect of growth hormone on adrenal function was investigated In the following experiments intact male rats were administered 0 9 mg porcine or monkey growth hormone for 2 days Two hours after the last injection the adrenals were removed from the control and growth hormone treated rats and were incubated in Krebs Ringer solution The adrenals of the growth hormone treated rats secreted slightly greater amounts of aldosterone than those of the control animals (Table IV) Whereas the porcine growth hormone did not affect the secretion rate of corticosterone there was a higher secretion rate of this corticosteroid in the adrenals of the rats treated with monkey growth hormone

Because of the difficulty in obtaining adrenals from intact

Table VI

EFFECT OF PLASMA FROM GROWTH HORMONE TREATED RATS ON THE *in vitro* SECRETION OF CORTICOSTEROIDS OF NORMAL ADRENALS

	Incubation medium	*Aldosterone* µg /g /hour	*Corticosterone* µg /g /hour
Intact rats	5 ml plasma—controls	13 9	[illegible]1 8
	5 ml plasma—growth hormone treated	15 0	42 8
Hypophysectomized rats			
Group A	6 ml plasma—controls	13 0	46 0
	6 ml plasma—growth hormone treated	15 3	42 5
Group B	10 ml plasma—controls	13 8	2[illegible] 3
	10 ml plasma—growth hormone treated	17 0	35 3

Table V

In vivo EFFECT OF GROWTH HORMONE ON ADRENALS OF HYPOPHYSECTOMIZED RATS

	Adrenal weight (mean)	*Incubation time*	*Aldosterone*	*Corticosterone*
	mg	hours	μg /g /hour	μg /g /hour
GROUP A				
Controls—hypophysectomized 4 days	16 0	3	13 6	35 7
Growth hormone treated*—hypophysectomized 4 days	14 6	3	19 8	27 6
GROUP B				
Controls—hypophysectomized 14 days	8 1	3	8 7	5 1
Growth hormone treated*—hypophysectomized 14 days	8 7	3	17 0	6 4

* 0 5 mg monkey growth hormone 475 6C daily for 3 days

eds Smith R W Jr Gaebler O H and Long C N H New York McGraw Hill)

Luft Dr Venning I would like to add some data regarding the relationship between the hypophysis and aldosterone excretion As I have already *mentioned* it is the free fraction of aldosterone and not the reduced metabolite that was studied Hypophysectomized patients in the first forty eight hours following operation had an increase in aldosterone excretion while the excretion of 17 hydroxy corticoids went down to extremely low values We also know that during the same period these patients had increased urinary volumes Furthermore Dr Hokfelt and I studied the aldosterone excretion in a number of patients with chromophobe adenoma of the hypophysis growing above the sella turcica and displacing the hypothalamus and of course also the third ventricle When we put these patients on a low sodium diet no increase in urinary aldosterone resulted in most of them while the same diet induced a significant change in normal subjects In addition in these patients the diurnal rhythm of 17 hydroxycorticoids in blood was absent All these data seem to point to a hypothalamic control of aldosterone production

Russell Fraser Dr Venning I was interested in your first series of experiments on the incubated glands showing that very high doses of growth hormone slightly increase the production of many steroids from the adrenals I was surprised that you should suggest that this might be due to contamination with ACTH It seems to be an extraordinary effect to get from ACTH i e an output of aldosterone as well as other steroids Might this not be another illustration of what might be called the polyglandular stimulating effect of growth hormone which in large doses seems to have a variety of glands as a target? I wonder whether in your *in vivo* experiments later it might not be somewhat like the parathyroid effect—only apparent if you have a great excess of growth hormone and if this was so one might expect the effect at a varying time after the starting of the large doses of growth hormone In our patients we have found that sodium retention was an early and a rather short lived effect and the other effect lasted very much longer One might by this means be able to discriminate between a direct and an indirect action

Venning We find that most preparations of ACTH with the exception of the U S P standard of Armour cause an increase in aldosterone secretion *in vitro* in human and rat adrenal gland tissue Of course the rise in the secretion rate of hydrocortisone is much greater than that observed with aldosterone

Li I understand Dr Venning that you have done experiments *in vitro* with ACTH using human adrenal gland tissue and got an increase of aldosterone but not as marked as corticosterone?

DISCUSSION

Anderson I congratulate the authors on this beautiful work and would like them to comment on how they would correlate certain other findings with their interpretation of the effect of growth hormone It has been shown by Ball and Davis that urinary aldosterone falls immediately following hypophysectomy but rises again if a partial ligature is placed on the vena cava (Ball W C and Davis J O (1957) *Amer J Physiol* **191** 329) In this condition you have *no growth hormone no ACTH* no pituitary *hormone of any kind to* stimulate the secretion of aldosterone Of course you might say that this hypophysectomized dog has his pineal and midbrain but we have found that after complete transection at the upper midbrain level in the dog the aldosterone in the adrenal venous blood is normal and the urinary excretion of aldosterone is normal

Venning It is very difficult of course to explain these findings The effect of growth hormone on aldosterone secretion appears to be independent from the effects that are noted in your particular experiments The hypothalamus and the pineal gland are intact in our rats and yet the administration of growth hormone causes a rise of some substance in the blood which is stimulating the secretion of aldosterone Of course we do not know the nature of this factor at the present time Farrell has shown that extracts of pineal glands contain a substance which stimulates aldosterone (Farrell G (1959) *Endocrinology* 65 239)

Beck I don t think we would propose either that this is synonymous with glomerulotropin My personal feeling is that it may be just one of the factors that influence aldosterone secretion it may not be the specific substance It should be emphasized that it may be that growth hormone undergoes some biological change on injection which allows it to become active and which we might not see if it were added directly to plasma *in vitro* There is evidence for that in other areas

Anderson It is unfortunate that we have nobody present at this colloquium to speak about the effect of growth hormone on cardio renal physiology it is conceivable that some changes in renal physiology might be involved in the excretion of aldosterone

Beck That is being done by us at present in humans and unfortunately so far we have studied only patients with abnormal renal function I do not think that this is a valid method of studying the direct actions of growth hormone on the kidney which certainly in Dr White s hands were very impressive (White H L (1955) *In* The Hypophyseal Growth Hormone Nature and Actions p 178,

mone gives the same drop in blood sugar as 3 m units of insulin These animals which responded to growth hormone have had their islet cells destroyed by alloxan so we feel that the hypoglycaemia is not due to release of insulin but is a direct effect of growth hormone

Beck There is a good deal of evidence from animal studies that this is not due to insulin release

Pearson We have given growth hormone intravenously in a dose of 5 mg to diabetic subjects normal subjects and hypophysectomized subjects with or without diabetes Blood sugar and unesterified fatty acid levels were measured at 15 30 60 120 and 180 minutes after the injection In all patients there was an initial decline in the blood sugar and unesterified fatty acid levels the maximum decline usually occurring at 30–60 minutes Following this decline the blood sugar level gradually returned towards the preinjection level at 2 and 3 hours whereas the unesterified fatty acids rose to very high levels at 2 and 3 hours In diabetic hypophysectomized subjects the higher the initial blood sugar level the greater was the decline in blood sugar level but in no case did severe hypoglycaemia develop after injection of growth hormone Insulin was withheld for a minimum period of 24 hours before injecting the growth hormone in the diabetic patients who were receiving insulin This growth hormone induced decline in blood sugar and unesterified fatty acid levels was observed in hypophysectomized juvenile type diabetic patients in whom there is reason to believe that little or no endogenous insulin can be produced

These observations suggest that the growth hormone induced decline in blood sugar and unesterified fatty acid levels is not due to insulin release from the pancreas Previous animal studies have indicated that this effect of growth hormone can occur in the absence of the pancreas Dr Anderson have you withdrawn insulin completely in your studies and still seen this effect?

Anderson I don t believe insulin needs to be present to obtain the hypoglycaemic effect with growth hormone As I mentioned we have obtained a significant hypoglycaemic effect in the hypophysectomized alloxan diabetic rat with amounts as low as 10 μg of growth hormone

Young Recently Manchester and I have investigated again the effect of growth hormone on the glucose uptake of isolated rat diaphragm and we found as others have done before that a clear *in vitro* effect of growth hormone is observed with diaphragm from hypophysectomized rats but not with that from normal rats (Manchester K L and Young F G (1959) *J Endocr* 18 381) This effect with diaphragm from hypophysectomized rats is not abolished by anti insulin serum With anti insulin serum present in the

Venning The greatest effect is observed with hydrocortisone corticosterone is also increased and aldosterone to a lesser extent

Li Dr Carstensen and Dr Burgers have recently reported that aldosterone was the predominant steroid detected in incubates of adrenals from the American bullfrog *Rana catesbeiana*, under the stimulation of bovine ACTH (Carstensen H, Burgers A C J and Li C H (1959) *J Amer chem Soc* 81, 4109) Some corticosterone was also found, but no cortisol or cortisone The ratio of aldosterone to corticosterone was 3 6 1 thus the aldosterone is apparently the major steroid stimulated by ACTH Perhaps the combination of ACTH and growth hormone is the best inducer of aldosterone secretion from the adrenal gland *in vitro* whether it is rat or human adrenal I am wondering if it would be worth while to carry out this experiment

Venning I think it would When we administer ACTH intramuscularly to normal individuals we invariably obtain a rise in urinary aldosterone

Beck There is one other explanation it may be a direct influence of ACTH on aldosterone synthesis at least in our hands My suggestion would be contradicted by your observations Prof Li The other possible mechanism would be that you are stimulating corticosterone which after all, is probably the substrate for aldosterone synthesis If you had more corticosterone around it might be that aldosterone synthesis would go on in association with ACTH but without being directly due to it

Anderson Dr Beck I should like to know more about your findings on hypoglycaemia How soon after the giving of growth hormone did you get hypoglycaemia and could you get it after every injection e g in the case of daily injections did you find hypoglycaemia each day or did it not persist? Also was the patient in a fasting condition when he became hypoglycaemic?

Beck These studies have been carried out in so called normal medical students Hypoglycaemia occurs between ten and twenty minutes after a single intramuscular injection of growth hormone usually after a 14 or 18 hour fast We do blood sugars every ten minutes and at the end of one hour the patient s blood sugar has returned to control levels We have not attempted to repeat it day by day but we have repeated it in the same subjects say at four five or seven day intervals and the effect has been present

Anderson We have found the same thing in hypophysectomized alloxan diabetic fasted rats in 25 minutes there is a fall in blood glucose which gives a linear dose response curve to 10 µg 30 µg and 90 µg of growth hormone (prepared by Wilhelmi for the National Institutes of Health Bethesda) the effect of 10 µg of growth hor

your 13 year old panhypopituitary in 2 injection periods his alkaline phosphatase seemed to go down Is that true ?

Beck That is quite true First of all he had an elevated alkaline phosphatase for reasons that we have never been able to explain It is still up $2\frac{1}{2}$ years later and he has had adequate amounts of vitamin D However in other pituitary dwarfs that we have treated for a short term period there has been no change in the alkaline phosphatase whereas when they are on prolonged therapy there is a rise I have no explanation for this curious finding

Sonenberg Some experiments we have made may be related to yours in that at times we have seen a fall in alkaline phosphatase after acute injections of growth hormone It may be related somehow to the period of fasting beforehand or the food intake but I am not sure about that

Beck We have made no observations on that

Luft Could the change observed in acute experiments with growth hormone be due to an increase in the extracellular fluid volume ?

Beck We like you have certainly measured extracellular fluid volume and total body water and there is an increase but I don t think it could be correlated with the fall in alkaline phosphatase in this patient

Rodriguez We did some experiments on the diabetogenic action of different preparations of growth hormone in force fed rats with mild alloxan diabetes with a fasting blood sugar of 150–200 mg and a glycosuria of not more than 100 mg in 24 hours We used bovine growth hormone monkey growth hormone prepared by Dr Raben and human growth hormone prepared by Dr Raben and Prof Li In the conditions of our experiment all of these hormones have more or less the same diabetogenic action when 1 mg per rat per day is given by subcutaneous injection during 4 days

Luft I would like to know if the members of the colloquium have noticed any change in the well being of patients who were given growth hormone We have some isolated observations in this respect In two of the patients in whom we did metabolic studies some psychical changes were noticed the patients became nervous and somewhat difficult to handle they also had some anorexia One member of the group who had an inactive rheumatoid arthritis complained of general malaise and some pain in the joints

Raben I have found on the contrary in the course of treating pituitary dwarfs that they have been more vigorous and active and quite happy and I have seen no evidence of any sort of deterioration Of course the doses involved are quite different—you used I think about ten times our dosage

medium the effect of growth hormone is clearly not due to the release of insulin bound in the tissues so a direct effect of growth hormone is much more likely We cannot reproduce this action of growth hormone with diaphragm from normal rats for some unexplained reason

Wilhelmi It is quite possible that some of these effects on early hypoglycaemia may be related to observations that have been reported recently by Winegrad and co workers and also by Jungas and Ball working with the epididymal fat pad *in vitro* (Winegrad A I Shaw, W N Lorens, F D W Stadie W C and Renold A E (1959) *J biol Chem* 234 1922 Jungas R L and Ball E G (1959) Amer chem Soc Meeting Boston Mass Abstr 61C) Here using relatively high doses of growth hormone (0 1–1 0 mg /3 ml of medium) both groups have observed that there is evidence of an increased utilization of glucose in the presence of growth hormone which is reflected in one instance by an increased output of $^{14}CO_2$ from uniformly labelled glucose and in another instance by alterations in the apparent RQ as Ball measures it and thirdly by direct measurement of the disappearance of glucose from the medium In our own laboratory a student Mr J N Fein has repeated these observations in the design of Dr Ball s experiments and he has seen this effect also that there is on incubation with growth hormone an increased uptake of sugar from the medium If this is an early effect of the hormone and if it is reflected in all of the adipose tissue to some extent then perhaps an insulin independent uptake of glucose may be observed Winegrad and his colleagues also saw that in epididymal fat tissue from alloxan diabetic rats the same effect of enhanced $^{14}CO_2$ production was observable so that here also there might be some reason for an increased glucose disappearance which could result in a mild order of hypoglycaemia shortly after administration of the hormone

Luft Altszuler and co workers studied the disappearance rate of [^{14}C]glucose in dogs and observed a decreased disappearance when growth hormone was given but in spite of this the peripheral utilization of glucose was increased (Altszuler *et al* (1959) *Amer J Physiol* 196 121) The implication would be that growth hormone in dogs increases the production of glucose by the liver

Beck Would anyone care to hypothesize that there is a fall in unesterified fatty acids ?

Pearson It is relatively small but it does appear to be significant in the studies that we have done

Beck We certainly believe that it is significant

Sonenberg Dr Beck you mentioned that you see the rise in alkaline phosphatase only on more prolonged injections and in

of Langerhans is the level of the blood sugar I would be interested in any comments on this question

Anderson I agree with you one hundred per cent!

Pearson Dr Rodriguez how do you explain the profound difference in the effects of growth hormone in the non diabetic and in the diabetic? Since one has functioning islet cells and the other has a marked diminution or perhaps absence of islet cells it would seem likely that the islet cells do something to protect the patient How do you eliminate the islet cells?

Russell Fraser Surely it is not the blood sugar level but the tissue sugar level that is effective?

Anderson Do you mean that it is the sugar level in the pancreatic tissue which stimulates the release of insulin?

Russell Fraser Yes

Anderson We have done a series of experiments using over 30 dogs in which we have studied the effect of various agents on the rise of insulin in the pancreatic venous blood and the only agent that causes a very significant rise in insulin is glucose If we raise the glucose level in the blood to 250–300 mg per cent we get more than a fivefold increase in the insulin content this is based on the assay of insulin in the blood Growth hormone prolactin ACTH and tolbutamide have no effect *per se* on the release of insulin

Russell Fraser Others have done experiments in which I think you will agree sulphanylurea infused into the pancreas does produce a rise in the insulin content of the efferent venous blood

Anderson Have they assayed the insulin in the pancreatic blood?

Russell Fraser Yes Dr Pfeiffer in Stuttgart has done that (Pfeiffer E (1959) *Proc III Congr int Diabetes Fed* Stuttgart Thieme) I would not think that it alters the main hypothesis in the slightest It is hard to believe how the level of sugar in the blood can have any effect if it does not produce that effect on the interior of a cell

Sonenberg Dr Beck has referred to the dissociation of the hyperglycaemic effect from the anabolic effect and I think this is consistent with the experience of many others We have recently demonstrated hyperglycaemia in hypophysectomized rats treated for a long period of time at the same time that they were demonstrating the usual increase in weight and it was our feeling that this had not been the experience in rodents before So I wonder if this so-called dissociation is not merely one of dose and if you give a sufficient amount of growth hormone for a sufficient period of time you might be able to demonstrate both in the same experimental situation

Russell It may be true that dosage is a factor Another point to be considered with respect to hypoglycaemic and hyperglycaemic

Luft Our patients in whom we carried out metabolic studies were given 10 mg of growth hormone daily for 12–16 days

Beck We have observed that on giving large doses of growth hormone to two dwarfs they became withdrawn and rather surly, where they had been smiling and pleasant before and they also developed very marked anorexia and we had great difficulty in getting the balance diet into them However with smaller doses over a long term I would agree with Dr Raben that they seem much happier

Raben In obese patients on low calorie diets as Dr Henneman also noted in one of his patients the appetite seems to diminish and I wonder whether this could be partly due to ketosis Do you think that some of your effects Dr Luft were related to ketosis?

Luft I do not think so Dr Raben You may have noticed from my slides that the increased excretion of ketone bodies which we observed in three patients out of four appeared during the initial part of the growth hormone period and then disappeared despite continued growth hormone administration the general deterioration of these patients occurred during the latter part

Pearson We have seen two situations where growth hormone seems to cause the patient to deteriorate very rapidly One was in the hypophysectomized diabetic patient already discussed We have also been interested in determining whether growth hormone would stimulate the growth of tumours Human growth hormone in doses of 5 mg /day has been administered to seven patients with osseous metastases from breast carcinoma who appeared to be improving following hypophysectomy In two patients there was a marked exacerbation of bone pain within two or three days which subsided when the growth hormone was withdrawn There was a concomitant rise in calcium excretion in these patients In the other five patients growth hormone was given for two to three weeks without producing any untoward effects We cannot interpret the significance of the deleterious effects of growth hormone in these two cancer patients

Dr Philip Henneman has had two patients with cranial pharyngiomata who developed signs of increased intracranial pressure while receiving growth hormone He felt that growth hormone may have stimulated the growth of this lesion

Loraine In the patients with mammary cancer treated with growth hormone was there any radiological evidence of deterioration or was the period of therapy too short to assess this?

Pearson These were very acute effects seen within a few days

Rodriguez One of our working hypotheses is that the action of growth hormone on the islets of Langerhans is not a direct one because we believe it is probable that the only stimulus for the islets

of Langerhans is the level of the blood sugar I would be interested in any comments on this question

Anderson I agree with you one hundred per cent!

Pearson Dr Rodriguez how do you explain the profound difference in the effects of growth hormone in the non diabetic and in the diabetic? Since one has functioning islet cells and the other has a marked diminution or perhaps absence of islet cells it would seem likely that the islet cells do something to protect the patient How do you eliminate the islet cells?

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Russell It may be true that dosage is a factor Another point to be considered with respect to hypoglycaemic and hyperglycaemic

effects of growth hormone is their time course When growth hormone is given to normal rats hypoglycaemia usually appears only rather slowly the maximum effect is seen at about 4–6 hours after growth hormone Hypoglycaemia of this type is not seen in animals previously treated with alloxan but instead hyperglycaemia is usual The alloxan diabetic animal need not be severely diabetic to exhibit this reversal of the effect of growth hormone but does show reduced pancreatic function For this reason I think that the relatively late hypoglycaemia must be related to the secretion or activity of insulin The more acute hypoglycaemia seen within a half hour or an hour such as that described by Dr Anderson may be quite different in origin

Another important consideration relates to the probable site of action of the hormone in producing its metabolic effects In the intact rat it is extraordinarily difficult to demonstrate any action of growth hormone on glucose tolerance This can be done with very large doses of hormone but even then the effect is minimal At the same time however profound changes must be taking place in the utilization of carbohydrate within the tissues as shown by reduction in the RQ and in the increased deposition of glycogen in muscle The term diabetogenic is probably not a good one to use to describe the metabolic effects of growth hormone because these are not readily reflected by changes in the blood sugar or in glucose tolerance curves The latter are secondary effects which are seen under particular conditions or during certain time intervals The so called diabetogenic effects—hyperglycaemia and reduced glucose tolerance—must in themselves be quite secondary to the primary action of the hormone within the tissues It may be better then to speak of growth hormone as inhibiting carbohydrate utilization rather than as diabetogenic Inhibition of carbohydrate metabolism may result from an increase in mobilization and use of fat but in the absence of evidence on this point I would prefer to think that the mobilization of fat was a consequence of the action of growth hormone on the metabolism of carbohydrate in adipose tissue This is a speculation of course I have some idea about what growth hormone may be doing in the tissues including adipose tissue but unfortunately I do not yet have any evidence on this point

HUMAN PITUITARY FOLLICLE STIMULATING HORMONE

I Clinical Effect of a Partially Purified Preparation

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A PARTIALLY purified human pituitary preparation of follicle stimulating hormone (HP FSH) was tested in 40 young amenorrhoeic women chosen at random from the out patient clinic of the Karolinska Hospital in Stockholm 14 had a primary amenorrhoea and 26 a secondary one (Gemzell Diczfalusy and Tillinger 1958 1959)

Assessment of clinical effect

In the assessment of the clinical effect of the hormone the following criteria were employed changes in ovarian size by pelvic examination inspection of the ovaries by culdoscopy or surgical exploration uterine biopsy and chemical determinations of urinary oestrone oestradiol 17β oestriol pregnanediol 17 ketosteroids and corticosteroids (17 ketogenic steroids) In some cases the luteinizing factor in the form of human chorionic gonadotropin (HCG) was administered immediately after HP FSH treatment It was thought that in order to obtain an ovulation by HCG one or several follicles had to be stimulated Consequently an ovulation obtained might indicate ovarian response to HP FSH

Evaluation of endometrial activity

The endometrium was evaluated as atrophic when no glandular mitosis was found and as proliferative and secretory (Table I)

Table I

EVALUATION OF ENDOMETRIAL ACTIVITY

	Proliferative			Secretory	
Atrophic	Weak	Moderate	Intense	Early secretory phase (preparedness)	Full secretory phase
No glandular mitosis	> 8*	< 8– >2	< 2	15th–18th day of cycle†	19th–28th day of cycle

* Number of glandular cross sections studied necessary to detect one mitosis (Tillinger and Westman, 1957)
† Basal vacuolization similar to the one found in normal cycles (Noye Hertig and Rock 1950)

Dose of HP-FSH

The HP FSH was administered intramuscularly in daily doses of 10 mg during a 10 day period The 10 mg dose was chosen as it produced a significant effect in a hypopituitary dwarf (18) while 1 mg per day had no effect and the doses of 2 and 5 mg per day only produced a slight increase in the proliferative activity of the endometrium Ten milligrams per day gave rise to a polycystic enlargement of the ovaries and a significant increase in the oestrogen excretion (Fig 1)

In a young woman with primary amenorrhoea (1) a daily dose of 5 mg during a 10 day period had no effect, while 10 mg per day during periods of similar length before and after produced a significant increase in ovarian size and oestrogen excretion

Repeated administrations of HP-FSH

Repeated treatment with HP FSH was performed in four women One woman with primary amenorrhoea (1) was treated seven times during one year and received altogether 710 mg of HP FSH A menstrual bleeding followed each treatment and the effect of the hormone on the ovarian size and the oestrogen excretion did not seem to change from one treatment to another

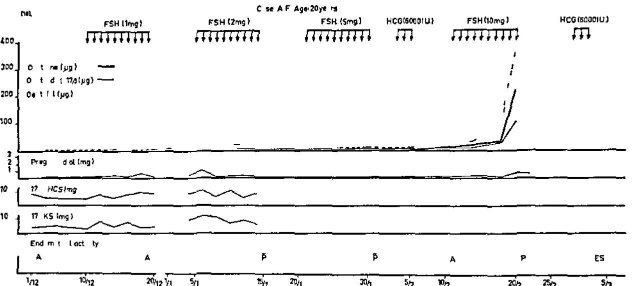


FIG 1 Endometrial activity and 48 hour urinary excretion of oestrogens pregnanediol 17 ketosteroids (17 KS) and corticosteroids (17 OHCS) *in a hypopituitary dwarf (18)* during treatment with different dose levels of HI FSH In this and subsequent figures biopsy findings are designated as follows A=atrophic P̄=weakly proliferative I =moderately proliferative P=intensely proliferative LS=early secretory S=secretory

Results

Following the administration of 10 mg of HP FSH during a 10 day period a polycystic enlargement of the ovaries was found in 27 out of 40 amenorrhoeic women (Table II) The urinary excretion of oestrogen increased in 16 of pregnanediol in 4 and of 17 ketosteroids and corticosteroids in 3 out of 30 women There was a group of 11 patients who did not show any response to HP FSH

Table II

EFFECT OF HP FSH (10 MG × 10) ON THE OVARIAN SIZE URINARY EXCRETION OF OESTROGENS PREGNANEDIOL 17 KS AND 17 OHCS IN AMENORRHOEIC WOMEN

	No of patients	*Increase*	*No change*
Ovarian size	40	27	2 + 11
Oestrogen excretion	30	16	3 + 11
Pregnanediol excretion	30	4	15 + 11
17 KS excretion	30	3	16 + 11
17 OHCS excretion	30	3	16 + 11

The effect of HP FSH on the endometrium is shown in Table III Of 27 women with atrophic endometrium before the

Table III

EFFECT OF HP FSH (10 MG × 10) ON THE ENDOMETRIUM OF AMENORRHOEIC WOMEN WITH DIFFERENT ENDOMETRIAL ACTIVITY BEFORE TREATMENT

Endometrial activity before FSH treatment	*No of patients*	*Endometrial activity after FSH treatment*					
			Proliferative			*Secretory*	
		Atrophic	*Weak*	*Moderate*	*Intense*	*Early*	*Full*
Atrophic	27	11	2	12	2		
Proliferative (weak)	3				1	2	
Proliferative (moderate)	[illegible]		1		1	1	4
Secretory (early)	1						1
Sum	38	11	3	12	4	3	[illegible]

treatment, 16 responded with proliferative activity while 11 did not show any endometrial change These 11 women were the same 11 reported in Table II They will be discussed later Fig 2 shows a typical response to HP FSH in a young woman

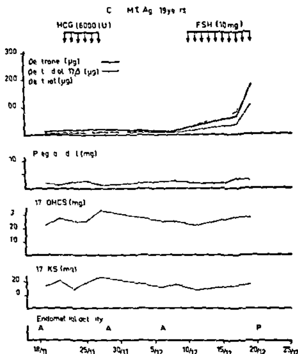


FIG 2 Steroid excretion (48 hours) and endometrial activity in a woman with secondary amenorrhoea during treatment with HP FSH

(24) with secondary amenorrhoea The ovaries were polycystic and enlarged the urinary excretion of oestrogen was increased and the endometrium changed from atrophic to moderately proliferative No change in pregnanediol, 17 ketosteroids and corticosteroids was observed A withdrawal bleeding appeared seven days after the last injection

Results

Following the administration of 10 mg of HP FSH during a 10 day period a polycystic enlargement of the ovaries was found in 27 out of 40 amenorrhoeic women (Table II) The urinary excretion of oestrogen increased in 16 of pregnanediol in 4 and of 17 ketosteroids and corticosteroids in 3 out of 30 women There was a group of 11 patients who did not show any response to HP FSH

Table II

EFFECT OF HP FSH (10 MG × 10) ON THE OVARIAN SIZE URINARY EXCRETION OF OESTROGENS PREGNANEDIOL, 17 KS AND 17 OHCS IN AMENORRHOEIC WOMEN

	No of patients	*Increase*	*No change*
Ovarian size	40	27	2 + 11
Oestrogen excretion	30	16	3 + 11
Pregnanediol excretion	30	4	15 + 11
17 KS excretion	30	3	16 + 11
17 OHCS excretion	30	3	16 + 11

The effect of HP FSH on the endometrium is shown in Table III Of 27 women with atrophic endometrium before the

Table III

EFFECT OF HP FSH (10 MG × 10) ON THE ENDOMETRIUM OF AMENORRHOEIC WOMEN WITH DIFFERENT ENDOMETRIAL ACTIVITY BEFORE TREATMENT

Endometrial activity before FSH treatment	*No of patients*	*Endometrial activity after FSH treatment*					
			Proliferative			*Secretory*	
		Atrophic	*Weak*	*Moderate*	*Intense*	*Early*	*Full*
Atrophic	27	11	2	12	2		
Proliferative (weak)	3				1	2	
Proliferative (moderate)	7		1		1	1	4
Secretory (early)	1						1
Sum	38	11	3	12	4	3	[illegible]

When HCG was administered to women pretreated with HP FSH an ovulation occurred, usually within 48 hours as indicated by the rise in pregnanediol excretion the fall in oestrogen excretion and the secretory reaction of the endometrium In an amenorrhoeic woman (21) HP FSH raised the level of urinary oestrogen from less than 10 μg /48 hours to more than 5 mg enlarged the ovaries to a diameter of 5–7 cm and changed the endometrium from atrophic to proliferative Following HCG treatment an ovulation occurred as indicated by the sharp rise in pregnanediol excretion and the fall in oestrogen excretion During HCG treatment the size of the ovaries increased further reaching a diameter of about 15 cm Biopsy was performed on the day after the last injection of HCG and revealed a secretory endometrium similar to the one found on the fourth postovulatory day At the same time a culdoscopy was done and one of the large follicular cysts was evacuated and about 750 ml of fluid was collected for chemical analysis (Fig 3) The follicular fluid contained small amounts of all the three oestrogens in about the same proportion as in the urine Following the evacuation of the cyst the pregnanediol excretion decreased Due to this fact and to the tremendous amount of pregnanediol found in the urine (140 mg /48 hours) it may be assumed that progesterone was formed not only in the corpus luteum but also in the follicular cyst Unfortunately, the follicular fluid was not tested for pregnanediol The elevated level of the corticosteroids was probably due to some progesterone metabolite possibly pregnanetriol The corticosteroids were determined as 17 ketogenic steroids and pregnanetriol is such a steroid Two weeks following the ovulation a menstrual bleeding appeared

Data of the 11 women who did not respond to HP FSH are presented in Table V Three had a secondary amenorrhoea and 8 a primary one Three cases (14 15 16) exhibited a typical syndrome of gonadal dysgenesis with retarded growth and male (M) sex chromatin pattern Eight of these women were operated upon and their ovaries inspected The histological examination revealed marked hypoplasia in one case

Of the 10 women with proliferative activity of the endometrium before treatment with HP FSH, 7 responded with a secretory reaction indicating that an ovulation had occurred (Tables III and IV) Three typical cases (7, 26 36) with Stein

Table IV

RESPONSE TO HP FSH (10 MG × 10) IN AMENORRHOEIC WOMEN WITH VARIOUS PROLIFERATIVE ACTIVITY OF THE ENDOMETRIUM BEFORE TREATMENT

No	Initials	Diagnosis	Total gonadotropins in urine	Endometrial activity	
				Before	After
4	L F	S A	normal	$\bar{P}$	P
5	E G	S A (LH deficiency?)	low	P	$\bar{P}$
6	I F	S A (LH deficiency?)	normal	P	$\bar{\bar{P}}$
7	A S	S A Stein Leventhal	normal	P	S
26	K A	S A Stein Leventhal	normal	$\bar{P}$	ES
27	S C	S A	normal	P	ES
36	E J	S A Stein Leventhal	low	P	S
38	S L	S A	low	P	S
42	C L	P A	normal	P	S
43	I N	S A Hirsutism	normal	$\bar{P}$	ES
48	K S	S A	normal	ES	S

S A = secondary amenorrhoea
P A = primary amenorrhoea

Leventhal syndrome belonged to this group they ovulated early during the treatment One amenorrhoeic woman (48) received HP FSH treatment immediately after a spontaneous ovulation The ovaries increased in size and the urinary excretion of oestrogen was elevated However the pregnanediol excretion decreased during the last days of the treatment and a menstrual bleeding appeared exactly two weeks after the calculated day of ovulation Two amenorrhoeic women (5 6) ovulated after treatment with HCG alone Following HP FSH administration they showed polycystic enlargement of the ovaries and endometrial proliferation It may be assumed that the menstrual disorder of these women was due to a deficiency of the luteinizing factor of the pituitary

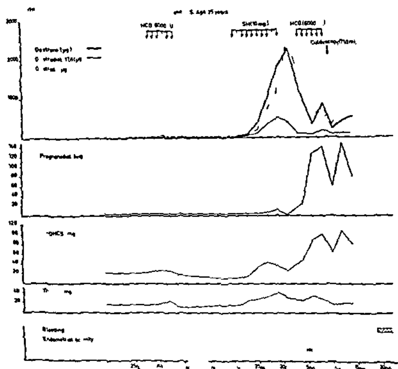


FIG 3 Steroid excretion (48 hours) and endometrial activity in an amenorrhoeic woman during treatment with HP FSH and HCG The single arrow indicates the evacuation of a follicular cyst and the collection of 7 0 ml of fluid

and complete lack of germinative tissue in the ovaries of the others Fig 4 shows the lack of response to HP FSH in a young woman (10) in whom surgical exploration revealed ovaries without germinative tissue

Two cases with typical Stein Leventhal syndrome (26 36) and one case (43) with an advanced hirsutism responded to HP FSH with marked increase in the urinary excretion of 17 ketosteroids and corticosteroids *Fig 5 shows the woman* with hirsutism HCG had no effect HP FSH increased the urinary excretion of 17 ketosteroids to 62 mg /48 hours and

Table V

SOME CLINICAL AND LABORATORY FINDINGS IN 11 AMENORRHOEIC WOMEN WHO DID NOT RESPOND TO HP FSH (10 MG × 10)

	8 (R S)	9 (K F)	10 (S N)	11 (M L)	1 (A A)	13 (G W)	14 (V S)	15 (I H)	16 (C L)	17 (S M)	18 (G K)
Diagno is	S A	S A	S A	P A	P A	P A	P A G D	P A G D	P A G D	P A	P A
Age	7	20	6	26	31	22	23	18	19	3	38
Sec sex characteristics	N	N	Inf	Inf	Inf	Inf	Inf	Inf	Inf	Inf	Inf
Uterine size (cm)	6	7	6 5	4	6	4	4	4	4	4	6
Endometrial activity	P	A	A	A	A	A	A	A	A	A	A
Total gonado-tropins	>50	>60<100	>10<40	0	>55<100	>50	>75	>60	>100	<30	>60<100
Blood pressure	12 /80	140/110	1 0/100	140/90	130/90	145/85	150/110	170/110	155/105	1 0/85	150/85
Congenital malformation	—	—	—	—	—	—	R G	R G	R G	—	—
Chromosomal sex	F	F	F	F	F	F	M	M	M	F	F
Stroma of ovarian type	+	+	+	+	+	+	0	0	—	—	—
Primordial follicles	+	+?	0	0	0	0	0	0	—	—	—
Leydig cells	0	0	+	+	0	0	+	+	—	—	—

S A = secondary amenorrhoea
P A. = primary amenorrhoea
G D = gonadal dysgenesis
R G = retarded growth.

Assay result wer 1 ul t d i term f a p vision 1 tand d prepared from th urine of men pausal and postm nopaus l wom n (HMG 0A) nd we e xp essed as HMG units/ 4 hrs An HMG unit i d fined as th acti ity of 1 mg of HMG 0A

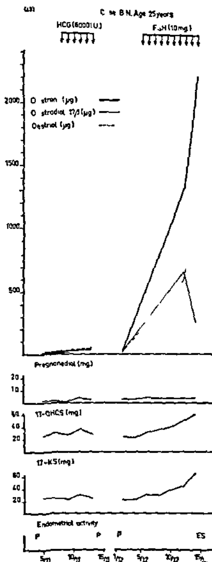


FIG 5 Steroid excretion (48 hours) and endometrial activity in a woman with secondary amenorrhoea and hirsutism during treatment with HP FSH.

the corticosteroids to 60 mg It may be assumed that these steroids orginate from the ovaries

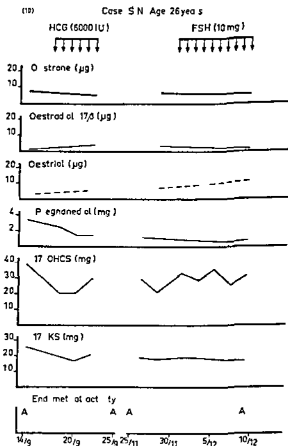


FIG 4 Steroid excretion (48 hours) and endometrial activity in a woman with secondary amenorrhoea and complete lack of germinative tissue in the ovaries during treatment with HP FSH

It was found in cases who ovulated on HP FSH alone that during a short period following ovulation there was no further increase in oestrogen excretion even if the administration of

μg /48 hours This level was still far less than was expected in similar cases Those results are only preliminary HP FSH was also administered to 4 pregnant women who in the sixteenth to the eighteenth weeks of pregnancy were operated upon for therapeutic abortion None of the ovaries of these women showed any signs of stimulation and histological examination revealed no follicular growth

In a few cases of amenorrhoea HP FSH was used to cure sterility Fig 7 shows a successful case (27) Following an HP FSH test to confirm an ovarian response a schedule was worked up to produce ovulations at certain points of time Two weeks after the second ovulation when the menstrual period failed to appear and the temperature following ovulation was still elevated it was suspected that fertilization had taken place Two weeks later pregnancy was confirmed by a positive Friedman test The woman was delivered 265 days after the day of ovulation of normal dizygotic twins weighing 2 7 and 3 3 Kg respectively

Discussion

It is apparent that HP FSH is a potent hormone when tested in human beings In amenorrhoeic women it produces enlarged polycystic ovaries and a great increase in urinary oestrogen When HP FSH was combined with the luteinizing factor in the form of HCG an ovulation occurred and a corpus luteum was formed which was capable of secreting substantial amounts of progesterone as shown by the change of the endometrium to secretory and by the greatly elevated output of pregnanediol As shown in one case the ova produced in this manner can be fertilized

The results seem to show also that in the human being stimulation of the follicular apparatus by HP FSH is essential for the effect of an ovulation inducing factor HP FSH by itself did not produce ovulation in ovaries without any oestrogen production as indicated by the atrophic endometrium In amenorrhoeic women with endometrial proliferation indicating a higher degree of ovarian activity, HP FSH alone was

HP FSH was continued It was thought that the newly formed corpus luteum was responsible for this effect and that eventually progesterone inhibited the oestrogen production

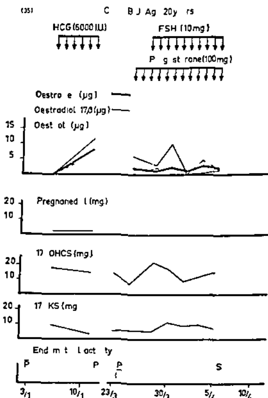


FIG 6 Steroid excretion (48 hours) and en dometrial activity in a woman with secondary amenorrhoea during treatment with HP FSH and progesterone

Progesterone in a daily dose of 100 mg was therefore ad ministered together with HP FSH to 5 amenorrhoeic women *It is apparent from Fig 6 (35)* that HP FSH did not increase the urinary excretion of oestrogen nor did it change the size of the ovaries The 4 other women responded in a similar way although in 2 of them the urinary oestrogen rose to about 50

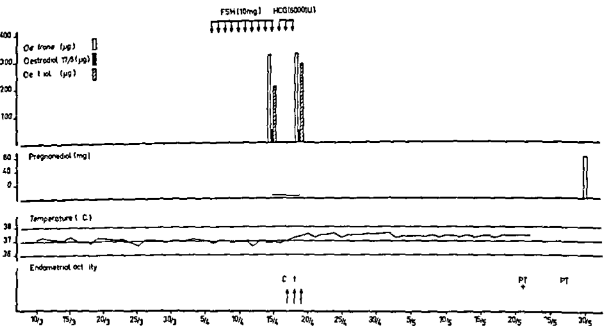
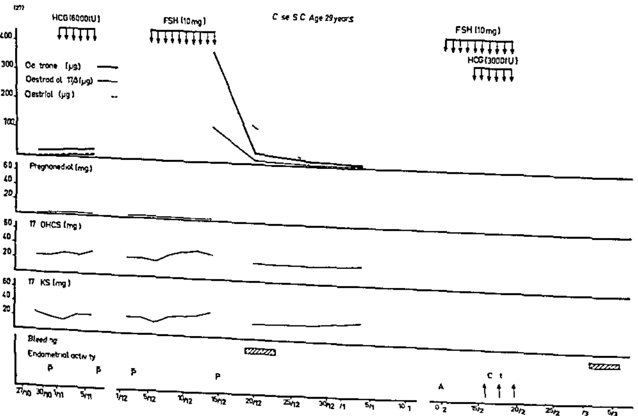


Fig. 7 *Steroid excretion* (48 hours) and endometrial activity in an amenorrhoeic woman (married for 6 years) *during* repeated treatment with HHFSH and HCG in order to produce *ovulation* The rise in body temperature indicates ovulation and the *positive* pregnancy test (PT) shows that fertilization has taken place


(27)
HCG (6000 IU)
FSH (10 mg)
Case S C Age 29 years
FSH (10 mg)
HCG (3000 IU)
Oestrone (µg)
Oestradiol 17β (µg)
Oestriol (µg)
400
300
200
100
Pregnanediol (mg)
17 OHCS (mg)
17 KS (mg)
60
40
20
Bleeding
Endometrial activity
P
P
P
P
A
C t
27/10 30/10 1/11 5/11 1/12 5/12 10/12 15/12 20/12 25/12 30/12 1/1 5/1 10/1 0 2 15/2 20/2 25/2 1/3 5/3

the other It would appear that patients with low or normal gonadotropin excretion will constitute the group of cases in whom further investigation of their ovarian function by means of an HP FSH test may be indicated

The marked increase in urinary 17 ketosteroid and corticosteroid excretion in 3 patients following treatment with HP FSH was not well understood One of these patients had a marked hirsutism and the other 2 a Stein Leventhal syndrome A similar increase in urinary 17 ketosteroids in 3 out of 16 patients with Stein Leventhal syndrome following treatment with hog or sheep FSH was reported by Keettel Bradbury and Stoddard (1957) They wondered whether FSH caused stimulation of the adrenal cortex In the three cases of this study the increase in 17 ketosteroids and corticosteroids occurred together with a marked increase in the urinary oestrogens Furthermore no effect on the adrenal cortex as indicated by a rise in 17 ketosteroids or corticosteroids was found when HP FSH was administered to the 11 women with ovarian failure Thus it may be assumed that 17 ketosteroids originate from the ovaries and that the polycystic ovaries found in cases with Stein Leventhal syndrome or in cases with hirsutism produce steroids normally not produced in the ovaries

It has been the general conception that progesterone exerts its influence on pituitary FSH through an inhibition of its release The results of this study indicate that another mode of action is possible Progesterone seems to have a local effect on the ovaries which protects them from the action of pituitary and exogenous FSH This may also be the mechanism in pregnancy where the insusceptibility of the ovaries to HP FSH may be due to the high concentration of progesterone in the body fluids

Summary

A partially purified preparation of human pituitary follicle stimulating hormone (HP FSH) was tested in 40 amenorrhoeic women A daily dose of 10 mg was administered during a 10-day period

able to induce ovulation Thus, it may be assumed that the response to HP FSH may be conditioned by the functional status of the ovaries

The polycystic condition of the ovaries and the high excretion rate of oestrogen indicate that a large number of follicles were stimulated following the HP FSH treatment This suggests that the dose administered was most probably higher than the normal requirement In this connexion it is of great interest that although a large number of follicles was stimulated only one or two follicles were brought to ovulation

In 11 out of 40 amenorrhoeic women HP FSH had no effect on the ovaries as far as ovarian size, endometrial activity and urinary excretion of oestrogen were concerned When the ovaries of 8 of these women were examined at operation they were found to be markedly hypoplastic and no germinative tissue was found at histological examination Thus it may be postulated that the presence of germinative tissue is necessary in order to obtain a response to HP FSH If so it would be tempting to assume that lack of HP FSH response is a reliable indicator of the virtual absence of functional germinative tissue

In 9 of the 11 women who did not respond to HP FSH a pathologically elevated urinary excretion of gonadotropins was found Subsequent examination at operation revealed a very hypoplastic or an entire absence of, germinative tissue of the ovaries This finding is in accordance with the well known observation that abnormally high urinary excretion of gonadotropins is associated with ovarian failure It follows from the present investigation that in such cases no further information can be expected from the administration of HP FSH On the other hand, a normal or low urinary gonadotropin excretion was found in all the 29 patients who responded to HP FSH Although these data are still preliminary they seem to indicate that a useful diagnostic approach to the evaluation of ovarian function in cases of amenorrhoea may be based on the assessment of ovarian response to HP FSH on the one hand and the urinary excretion of gonadotropins on

HUMAN PITUITARY FOLLICLE-STIMULATING HORMONE

II Preparation of a Highly Active Fraction

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THE partially purified hormone preparation used in the clinical experiments (Gemzell Diczfalusy and Tillinger 1958 1960) has been further purified by one of us (Roos) by means of ion exchange chromatography and zone electrophoresis

Samples of 200–225 mg of the crude preparation were stirred in a centrifuge tube containing 0 004 M sodium citrate buffer pH 5 2 Some insoluble material was centrifuged off and the pH and conductivity of the supernatant were adjusted to correspond to those of the buffer The solution (about 40 ml) was applied to a column (2 5 × 25 cm) packed with carboxymethyl (CM) cellulose in equilibrium with the citrate buffer and the column was operated at a rate of 24 ml /hour Under these conditions the follicle stimulating activity was found in the material passing unretarded through the column The weight of this fraction was 20–22 mg after dialysis and lyophilization No significant activity was found in the adsorbed material which was eluted with 0 8 M sodium citrate buffer of pH 6 0

The CM cellulose chromatography gave quite reproducible results and for the next purification step which was chromato graphy on diethylaminoethyl (DEAE) cellulose it was pos sible to use the unretarded fraction directly without prior dialysis lyophilization and activity determination To the anion exchange resin which was in equilibrium with 0 004 M sodium citrate buffer pH 5 8 either a volume of the active fraction (pH being adjusted to 5 8) containing 40–45 mg

The following effects were noticed

(1) Polycystic enlargement of the ovaries and a highly elevated level of urinary oestrogens

(2) Following repeated administrations the same ovarian response was found

(3) Human chorionic gonadotropin (HCG) induced ovulation in women pretreated with HP FSH, usually within 48 hours

(4) Women without ovarian response showed either complete lack of germinative tissue or marked hypoplasia of the ovaries

(5) In cases with Stein Leventhal syndrome ovulation was induced by HP FSH alone usually within 8 days of treatment

(6) In two cases with Stein Leventhal syndrome and one case with hirsutism a marked increase in the urinary 17 ketosteroids and corticosteroids was found

(7) The ovarian response to HP FSH was blocked by simultaneous administration of progesterone

(8) No ovarian response was found in pregnant women following administration of HP FSH

(9) In one amenorrhoeic woman a normal pregnancy was obtained following the combined treatment with HP FSH and HCG

(10) No ovarian response was found in amenorrhoeic women with a pathologically elevated excretion of gonadotropins while all cases (except two) with a low or normal excretion rate responded

REFERENCES

GEMZELL C A DICZFALUSY E and TILLINGER K G (1958) *J clin Endocr* 18 1333

GEMZELL C A DICZFALUSY E and TILLINGER K G (1959) *Acta obstet gynec scand* 38 465

KEETTEL W C BRADBURY J T and STODDARD F J (1957) *Amer J Obstet Gynec* 73 954

NOYES R W HERTIG A T and ROCK J (1950) *Fertil and Steril* 1 3

TILLINGER K G and WESTMAN A (1957) *Acta endocr (Kbh)* 25 113

doses corresponding to 0 010–0 200 mg were taken for each rat to be assayed

The results of an assay on a weight basis are summarized in Table I

Table I

FSH ACTIVITY OBTAINED BY FRACTIONATION OF A PITUITARY CONCENTRATE (459) ASSAYED AGAINST THE HUMAN MENOPAUSAL GONADOTROPIN STANDARD (HMG 20A)

Material	Number of rats	Total dose (mg)	Average ovarian weight (mg)	Activity times HMG 20A standard
Starting material	5	0 100	51	61 ×
	9	0 [illegible]00	80	
Active material from				
CM-cellulose column	5	0 0[illegible]	[illegible]4	2[illegible]1 ×
	5	0 100	105	
DEAE cellulose column	6	0 010	33	[illegible]08 ×
	6	0 025	61	
	6	0 100	102	
electrophoresis	8	0 010	94	2040 ×
	8	0 040	150	

As seen from Table I the fractionation procedure outlined here gives a product which is more than 30 times as active as the preparation used in the clinical study The procedure is however still under development and the results should be considered as preliminary

Papers dealing with the purification of human FSH have been published by Li (1958) Steelman Segaloff and Mays (1958) and Steelman Segaloff and Andersen (1959) Li measuring the activity in slope units described a procedure giving a product with an activity of approximately 600 units Using slope units the electrophoretically purified fraction in Table I seems to have an activity of about 5 000 units Steelman Segaloff and Andersen (1959) reported in an abstract preparations 40–50 times as active as the Armour Standard FSH 264–151X Since our material was assayed against another standard a comparison of the activities is not possible No physiochemical investigations have yet been reported

protein or the same amount of lyophilized material dissolved in the buffer, was therefore added With a flow rate of 18 ml /hour, some inactive material was found to pass straight through the column, the follicle stimulating hormone (FSH) being adsorbed Stepwise increase in buffer concentration at a constant pH of 5 8 gave an active fraction at a concentration of 0 02 M The yield of active material after dialysis and lyophilization was 20–25 mg corresponding to about 5 per cent of the starting material

The active fraction was submitted to zone electrophoresis in a vertical column (1 × 95 cm) packed with a copolymerisate of polyvinyl chloride and polyvinyl acetate in 0 05 M veronal buffer pH 8 6 The material was continuously removed from the column by the method of Porath Lindner and Jerstedt (1958) The current was 5 mA during 48 hours at an applied voltage of 1 100 v The material distribution was determined by the Folin Lowry reaction and on this basis the fractions were pooled into larger units which were dialysed and lyophilized The electropherogram showed three incompletely separated peaks the most basic of which was found to contain the follicle stimulating activity The yield from 19 mg was about 1 5 mg and the overall yield after chromatography and electrophoresis was about 0 5 per cent as measured by weight

The follicle stimulating potency was estimated by the ovarian augmentation test of Steelman and Pohley (1953) The dose of human chorionic gonadotropin (HCG) administered to each assay rat was 18 I U giving an average ovarian weight of 25 mg The activity of a fraction was measured against the international menopausal gonadotropin standard (HMG 20A)

Samples of the lyophilized fractions from the different purification steps were assayed routinely in the following way The samples were dissolved in 8 ml of 0 85 per cent sodium chloride solution and the absorption at 284 mμ was determined using 1 cm quartz cells On the assumption that 1 mg protein per ml gives an optical density of 1 0 at this wavelength

ethinyl nor testosterone (nor-ethisterone) which was kindly supplied to us by Schering A G Berlin We administered nor-ethisterone enanthate in relatively large doses to postmenopausal subjects with recurrent or metastatic mammary carcinoma and found that a marked fall in urinary gonadotropin levels occurred It would therefore appear that this compound at least in postmenopausal subjects can act as a pituitary inhibitor as far as gonadotropin secretion is concerned

Gemzell We have some preliminary results on the urinary excretion of this preparation which are very similar to those which you have obtained We administered intramuscularly 50 mg of this partially purified preparation to two hypophysectomized women and the recovery from the urine was very low probably about 5 per cent the activity was obtainable for at least 6–7 days following injection

Loraine Have you noticed any difference in response to FSH between patients with primary amenorrhoea on the one hand and with secondary amenorrhoea on the other?

Gemzell As yet we have not enough material to be able to show any differences The response to FSH varies very much from one case to another In one case we get a response of oestrogens of 400–500 µg /48 hours and in the next similar ca e the elevation goes up to several mg At present we are unable from the clinical findings in the patient to predict the effect

A B Houssay The increase in 17 ketosteroids in some of Dr Gemzell s patients raises a very interesting point i e that gonadotropins may act upon the adrenals but I do not know if this has been found in the human I think that there is experimental evidence that it happens in some pathological conditions In 1952 we were working on adrenal tumours produced by castration in rats and 30 per cent of our castrated rats developed oestrogenic adrenal tumours (Houssay B A Houssay A B Cardeza A F Foglia V G and Pinto R M (1953) *Acta physiol lat amer* 3 125) When we removed the pituitary the oestrus disappeared from these rats with adrenal tumours If we then gave them a mixture of pregnant mare serum gonadotropin and human chorionic gonadotropin the oestrus reappeared but if we administered ACTH instead the oestrus failed to reappear We thought that this proved that in certain pathological conditions gonadotropin may act upon the adrenals

Gemzell I do not know of any experiments where FSH has been used in an attempt to stimulate the human adrenals but certainly there have been many investigations on the effect of human chorionic gonadotropin on the adrenal cortex Dr Borell in our clinic

Summary

A highly active follicle stimulating fraction has been prepared from human pituitaries The fractionation, from a previously described concentrate was achieved by ion exchange chromatography and zone electrophoresis

Acknowledgments

We are indebted to Professor A Tiselius for his kind interest in this work

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REFERENCES

GEMZELL C A DICZFALUSY E and TILLINGER, K G (1958) *J clin Endocr* 18 1333

GEMZELL C A DICZFALUSY E and TILLINGER K G (1960) *Ciba Found Coll Endocr* 13, 191 London Churchill

LI C H (1958) *Proc Soc exp Biol (N Y)* 98 839

PORATH J LINDNER E B, and JERSTEDT S (1958) *Nature (Lond)* 182 744

STEELMAN S L and POHLEY F M (1953) *Endocrinology* 53 604

STEELMAN S L SEGALOFF A and ANDERSEN R N (1959) *Fed Proc* 18 330

STEELMAN S L SEGALOFF A and MAYS M G (1958) *Arch Biochem* 78 262

DISCUSSION

Loraine I wonder if Dr Gemzell has any information on the recovery of gonadotropins from urine following the administration of FSH Recently my colleagues and I administered approximately 900 HMG units of human FSH to a patient with primary amenorrhoea We found that this material produced a rise in urinary oestrogen excretion but no rise in urinary pregnanediol excretion When we conducted recovery experiments for gonadotropins in urine we found the yield to be low in that less than 5 per cent of the administered material was recovered over a period of seven days following injection of FSH We were also unable to detect the presence of gonadotropic activity in blood immediately following the injection of FSH and accordingly concluded that the rate of disappearance of gonadotropins from the circulation was very rapid

Regarding the effect of progestational substances on gonadotropin excretion we have recently had experience with the compound

17 ketosteroids might be the two glands adrenal and ovary Many years ago we determined the androgenic potency of the urinary extract by biological test and in the guinea pig given chorionic gonadotropin found very large androgenic potency We also found androgenic potency in ovarian extracts from injected guinea pigs using the castrated cock s comb test whereas no androgenic activity was found in ovarian extracts from control animals

Gemzell I have no experience of the use of human gonadotropins in animal experiments but all these women have been treated with human chorionic gonadotropin one or two months before FSH treatment It has been suggested that it is possible to produce ovulation with the luteinizing effect only In these 40 women there were only two who responded with an increase in pregnanediol excretion secretory reaction of the endometrium and ovulation only one of the 40 women responded with an elevation of the urinary 17 ketosteroids That was a woman with the Stein Leventhal syndrome where one would expect the synthesis of steroids to be disturbed

Malinow Dr Gemzell could fibrinogen levels be followed in these patients? We have confirmed some of Gillman s early work on fibrinogen levels in rats and found that female rats have lower fibrinogen levels than male rat these differences disappear after castration (Torres H N Malinow M R and Mirochnik L (1959) Unpublished data Gillman T and Naidoo S S (1958) *Endocrinology* 62 92) We also found that fibrinogen levels undergo a cyclic change they are lower during oestrus and higher during di oestrus Because we are interested in atherosclerosis we wonder whether that could be another way of protecting women from coronary occlusion I think that Dr Gemzell s patients are very suitable for this investigation and I would be very interested to know what the results would be

Gemzell I have no results on this but I agree with Dr Malinow that this may be very close to the problem to stimulate the ovaries and induce ovulation

Venning Dr Gemzell have you analysed the follicular fluid in the cysts of patients with the Stein Leventhal syndrome for andro genic material?

Gemzell We have not done that yet but we plan to do so

Raben Have studies similar to those which you have done on patients been done with animal FSH combined with chorionic gonadotropin?

Gemzell Such studies have been carried out especially by Scandi navian gynaecologists Rydberg and Oestergaard in Copenhagen (Rydberg E and Oestergaard E (1930) *Acta obstet gynec scand*

has shown that there are some effects although the dose of 150 000 units of human chorionic gonadotropin in a single administration was very high The question may be raised if this large dose by itself could produce changes that may activate the adrenal in an unspecific way

Corner Dr Gemzell in his beautiful series of experiments is clarifying very sharply the successive actions and relative effects of the various pituitary hormones on ovarian function Now what more can be said about the criteria for ovulation? In the one case in which pregnancy ensued the timing of the story and Dr Gemzell s success in predicting the course of it would certainly indicate that an ovum was shed as a result of the treatment I wonder however whether the appearance of a secretory reaction in the endometrium is proof of the actual discharge of a viable and fertilizable ovum. Theoretically the appearance of a secretory endometrium could result from alterations in the function of the follicle without rupture It is theoretically quite possible that progesterone may be formed in the ovary as a result of such a treatment without the discharge of an ovum It may be that the failure to secure pregnancy in certain cases even after these apparent signs of ovulation had occurred was due to the luteinization of an unruptured follicle

Gemzell I agree with Prof Corner that it may be very difficult to know whether this change in the endometrium is due to ovulation or due to progesterone eventually produced in the large follicles We find it very difficult to believe that a small corpus luteum alone could produce progesterone equivalent to 70 mg of pregnanediol per day In many of these cases where we found no indications that ovulation had occurred there were changes in the endometrium which were not usually found in the normal cycle We have speculated as to whether these changes in the endometrium were due to the high production of oestrogens or to other steroid metabolites We have not regularly used the increase in temperature but I think that may be one indication that ovulation has occurred

Morato Manaro I am very interested in Dr Gemzell s results on injection of patients with human chorionic gonadotropin I have done experiments to determine the amount of 17 ketosteroids in the urine of control guinea pigs in one series the average amount was 454 μg /day/guinea pig in normal guinea pigs injected with 50 units/day of chorionic gonadotropin during 16 days we got an average of 929 μg /day/guinea pig but in two castrated animals injected with chorionic gonadotropin an average of 828 μg /day/guinea pig was obtained The adrenal gland stimulated by the chorionic gonadotropin might produce 17 ketosteroids and in the normal guinea pig injected with chorionic gonadotropin the origin of the

17 ketosteroids might be the two glands adrenal and ovary Many years ago we determined the androgenic potency of the urinary extract by biological test and in the guinea pig given chorionic gonadotropin found very large androgenic potency We also found androgenic potency in ovarian extracts from injected guinea pigs using the castrated cock s comb test whereas no androgenic activity was found in ovarian extracts from control animals

Gemzell I have no experience of the use of human gonadotropins in animal experiments but all these women have been treated with human chorionic gonadotropin one or two months before FSH treatment It has been suggested that it is possible to produce ovulation with the luteinizing effect only In these 40 women there were only two who responded with an increase in pregnanediol excretion secretory reaction of the endometrium and ovulation only one of the 40 women responded with an elevation of the urinary 17 ketosteroids That was a woman with the Stein Leventhal syndrome where one would expect the synthesis of steroids to be disturbed

Malinow Dr Gemzell could fibrinogen levels be followed in these patients? We have confirmed some of Gillman s early work on fibrinogen levels in rats and found that female rats have lower fibrinogen levels than male rats these differences disappear after castration (Torres H N Malinow M R and Mirochnik L (1959) Unpublished data Gillman T and Naidoo S S (1958) *Endocrinology* 62 92) We also found that fibrinogen levels undergo a cyclic change they are lower during oestrus and higher during di oestrus Because we are interested in atherosclerosis we wonder whether that could be another way of protecting women from coronary occlusion I think that Dr Gemzell s patients are very suitable for this investigation and I would be very interested to know what the results would be

Gemzell I have no results on this but I agree with Dr Malinow that this may be very close to the problem to stimulate the ovaries and induce ovulation

Venning Dr Gemzell have you analysed the follicular fluid in the cysts of patients with the Stein Leventhal syndrome for androgenic material?

Gemzell We have not done that yet but we plan to do so

Raben Have studies similar to those which you have done on patients been done with animal FSH combined with chorionic gonadotropin?

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19, 222) and Westman and co workers in Stockholm (Claesson L Hogberg B, Rosenberg T and Westman A (1948) *Acta endocr* (*Kbh*) 1, 1) have done a great deal of research using mainly the follicle stimulating activity from pregnant mare serum followed by human chorionic gonadotropin It was possible in some cases to obtain a polycystic enlargement of the ovaries by the animal preparation, but I do not think that it was possible to repeat the effect. As Oestergaard has shown the animal hormones produce antihormones which prevent further effect (Oestergaard E (1942) Antigonadotropic Substances Copenhagen Munksgaard) I don t know of any patient in whom ovulation has been produced by such treatment

Russell Fraser Dr Gemzell have you used your preparations in completely hypophysectomized patients or in patients in whom you believe that the pituitary is deficient in various secretions as well as its gonadotropic function and did they respond similarly ?

Gemzell We have done it only in those few experiments where we were interested to see the excretion of hormone In one young girl who was a hypopituitary dwarf we used it mainly for testing the dose This girl had an accident during childhood with resulting calcification in her pituitary and did not excrete any gonadotropins Her basal metabolic rate and excretion of 17 ketosteroids were very low We obtained a normal response in this girl in other words her ovaries responded despite the fact that there had never been any menstruation

Bottari I was surprised both by the small amount of hormone recovered in the urine after injection of FSH in hypophysectomized patients and by the length of time necessary for the complete urinary elimination of FSH Thus I wonder how far urinary gonadotropin estimations reflect variations in the blood concentration The second point I would like to raise is whether peaks observed using 24 or 48 hour collections of urine are not merely a general reflection of the hormone elimination without any relationship in time to what is happening at a definite moment in the blood

Gemzell I think that Dr Loraine s and my own experiences were very similar We followed these patients for seven days and the urine was collected in 48 hour samples The amount of gonadotropins was in each case very small It was just enough to show activity

Loraine The low yield in recovery experiments of this type is not peculiar to gonadotropins but is found with other hormones as well For example when oestradiol 17β is administered to male subjects or to postmenopausal or amenorrhoeic women only 20 per cent appears in the urine as oestradiol 17β oestrone and oestriol

SOME OBSERVATIONS ON THE CLINICAL VALUE OF PITUITARY GONADOTROPIN ASSAYS IN HUMAN URINE

J A Loraine

Clinical Endocrinology Research Unit (Medical Research Council)
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The estimation of gonadotropins in urine is the only satisfactory direct measure of pituitary activity which is at present available in clinical investigations Such estimations have already been shown to be helpful in differentiating between the various types of gonadal failure (Klinefelter Reifenstein and Albright 1942 Pedersen Bjergaard and Tønnesen 1951) in the diagnosis of testicular tumours (Hamburger 1946 1958) and in the management of patients with mammary carcinoma (Loraine 1958*a* and *b* Loraine Strong and Douglas 1957) The main topics discussed in this paper are firstly the relationship of gonadotropin estimations to the treatment of recurrent or metastatic mammary carcinoma by bilateral adrenalectomy and bilateral oöphorectomy and secondly the excretion of urinary gonadotropins in patients with seminoma of the testis A short section is also included on the relationship between urinary gonadotropin and urinary oestrogen excretion in three subjects during the normal menstrual cycle One of these subjects was artificially inseminated during the period of study and the assay findings following insemination are described

Urinary gonadotropins were estimated by the method of Loraine and Brown (1959) Results are expressed in terms of a standard prepared from menopausal and postmenopausal urine (HMG 20A)* and are not corrected for losses incurred

* The letters HMG stand for human menopausal gonadotropin One HMG unit is the amount of activity present in 1 mg of HMG-20A. The biological characteristics of HMG have been described elsewhere (Loraine and Brown 1956 Loraine 1958*b* Borth Lunder and Lunenfeld 1959)

during the performance of the method In assays conducted in patients with mammary carcinoma and seminoma only the mouse uterus test was used as the end point of the bioassay as noted previously (Loraine, 1958b) this method is not specific for either follicle stimulating hormone (FSH) or interstitial cell stimulating hormone (ICSH) but measures what may be conveniently described as the 'total gonadotropic activity of human urine In some of the studies during the normal menstrual cycle parallel assays on the same urine samples were performed by the mouse uterus and hypophysectomized rat prostate tests the latter method is generally believed to be specific for ICSH activity (Greep Van Dyke and Chow, 1942 McArthur, 1952, Loraine and Diczfalusy, 1958)

Estimations of oestradiol 17β oestrone and oestriol during the normal menstrual cycle were conducted by the method of Brown (1955) For urinary pregnanediol determinations the technique of Klopper Michie and Brown (1955) was employed

Urinary gonadotropin assays in relation to the treatment of recurrent and metastatic mammary carcinoma by bilateral adrenalectomy and oophorectomy

Estimations before adrenalectomy and oophorectomy

Gonadotropin assays were performed in 32 patients *prior to* adrenalectomy and oophorectomy The time of observation before operation was variable and the number of readings in *individual subjects ranged from a minimum of 1 to a maximum* of 11 Following the operation patients were classified in terms of their response into two groups designated respectively remission and no remission details of the method of clinical classification have been described elsewhere (Strong 1958) It must be emphasized that in all patients bilateral adrenalectomy and oophorectomy were performed at a relatively late stage of the disease Previous forms of therapy

had included irradiation of the ovaries and administration of oestrogens and androgens

Results of assays before adrenalectomy and oöphorectomy are presented in summary form in Table I

Table I

GONADOTROPIN EXCRETION BEFORE BILATERAL ADRENALECTOMY AND OOPHORECTOMY

Group	*Number of patients*	*Number of observations*	*Mean gonadotropin excretion (HMG units/24 hrs)*	S D *log*	*Range—HMG units/24 hrs* ($P = 0\ 95$)
Remission	11	41	55	0 33	13–240
No remission	21	63	42	0 41	8–263

Analysis of significance

	t	P
Remission *v* no remission	1 56	0 2–0 1

Owing to the relatively wide variation in gonadotropin excretion in the various groups of patients the significance of the results shown in this and subsequent tables was tested in the following way as recommended by Gaddum (1945) The excretion values in individual patients were converted into logarithms The mean and standard deviation of each group of logarithms were then calculated Column 4 gives the gonadotropin excretion corresponding to the mean of the logarithms in each group and is thus the geometric mean of the observations Column 5 gives the standard deviation of the logarithms The figures in column 6 are derived from those in column 5 and give the range within which it is expected that 95 per cent of the results may be expected to be found

It will be noted (Table I) that although the mean gonadotropin excretion in the group designated remission was higher than that in the group designated no remission the difference between these values fell just short of significance ($P = 0\ 2 - 0\ 1$)

Estimations after adrenalectomy and oophorectomy

Gonadotropin assays were performed in 37 patients following bilateral adrenalectomy and oophorectomy Again, the period of observation varied considerably and the number of readings in individual patients ranged from a minimum of 1 to a maximum of 22 Where possible, serial determinations were made at 6 weekly intervals after the operation At these times consecutive urine samples were collected over a period of 4 days, the urine was extracted in 48 hour pools and one *gonadotropin assay was performed on each pool*

The effect of bilateral adrenalectomy and oophorectomy on gonadotropin excretion was shown to be variable in that after the operation, the excretion values might change little might rise markedly or might fall In patients in whom adrenalectomy was followed by a remission of disease the gonadotropin excretion tended to remain high or to rise, while in subjects in whom the disease failed to remit excretion values remained low or fell Typical examples of the different patterns of excretion following bilateral adrenalectomy and oophorectomy are presented in Figs 1 and 2

A postmenopausal subject M W , aged 68 (Fig 1) was treated in 1953 by simple mastectomy and radiotherapy for a carcinoma of the right breast In 1955 she was treated for recurrences on her chest wall first with ethinyl oestradiol and later with testosterone phenyl propionate neither form of therapy produced any striking effect on the progress of the disease In March 1957 she was subjected to bilateral adrenalectomy and oophorectomy This form of treatment resulted in a remission of her disease which lasted for approximately 18 months

It will be noted that before adrenalectomy the gonadotropin excretion was low ranging from 15 to 30 HMG units per 24 hours Some two months after the operation the readings started to rise and for the next 14 months remained in the range 100 to 280 HMG units per 24 hours The period of high gonadotropin excretion coincided with the period of remission of disease

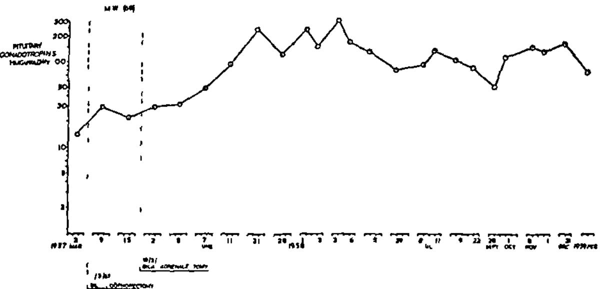


FIG 1 The effect of bilateral adrenalectomy and oöphorectomy *on urinary* gonadotropin excretion in a patient with mammary carcinoma *Following the operation* this patient showed a remission of disease of approximately 18 months duration

Another postmenopausal subject M R, aged 58 (Fig 2) was subjected to radical mastectomy in 1951 and remained well for the following 5 years In 1956 she became breathless on exertion and was found to have extensive mediastinal glandular enlargement and metastatic deposits in both lungs She was treated consecutively with stilboestrol dipropionate and cortisone acetate but after neither form of therapy was any beneficial effect noted In December, 1957 bilateral

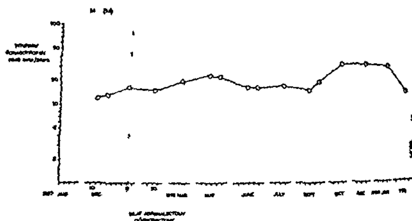


FIG 2 The effect of bilateral adrenalectomy and oöphorectomy on urinary gonadotropin excretion in a patient with mammary carcinoma This patient failed to respond to this form of therapy

adrenalectomy and oophorectomy were performed a remission did not ensue and the patient died from extension of her disease in March 1959

It will be noted that prior to the operation the gonadotropin excretion was low, being below 20 HMG units per 24 hours In the 14 months following the operation readings showed little or no tendency to rise remaining in the range 15–30 HMG units per 24 hours

A summary of the results obtained following adrenalectomy and oophorectomy is shown in Table II It will be noted that there was a highly significant difference between the mean

Table II

GONADOTROPIN EXCRETION AFTER BILATERAL ADRENALECTOMY AND OÖPHORECTOMY

Group	*Number of patients*	*Number of observations*	*Mean gonadotropin excretion (HMG units/ 24 hrs)*	S D *log*	*Range—HMG units/ 24 hrs (P = 0 95)*
Remission	14	105	62	0 39	11–355
No remission	23	120	34	0 35	7–178

Analysis of significance

	t	*P*
Remission *v* no remission	5 20	<0 001

gonadotropin excretion in the two groups of patients ($P <$ 0 001)

Comment

Previous work has shown that gonadotropin assays in patients with mammary carcinoma may be useful in assessing the completeness or otherwise of a hypophysectomy and in predicting the response to therapy with oral stilboestrol (Blackburn *et al* 1956 Loraine and Strong 1958 Loraine Strong and Douglas 1957) The present study has demonstrated that following bilateral adrenalectomy and oophorectomy a high gonadotropin excretion is usually associated with a remission of disease while a low excretion generally occurs in patients who fail to respond to the operation

Prior to adrenalectomy and oophorectomy gonadotropin excretion tended to be higher in patients who showed a subsequent remission than in those who failed to respond However as shown in Table I the difference between the mean excretion values in the two groups of patients was not significant *This may well have been due to the fact that* before adrenalectomy most of the patients in this series had been treated in ways which may well have influenced both their

pituitary function as judged by urinary gonadotropin assays and their subsequent response to adrenalectomy It is therefore possible that had adrenalectomy been performed at an earlier stage in the evolution of the disease, gonadotropin assays in urine might have proved of considerable value in selecting the patients who might have been expected to benefit from this form of treatment

Urinary gonadotropin assays in seminoma of the testis

Assays have been performed in nine patients with seminoma of the testis following treatment by unilateral orchidectomy In eight of these subjects serial readings commenced within one month of the operation and continued for periods ranging from 2 weeks to 5 months The remaining patient presented

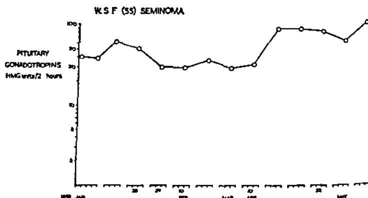


FIG 3 Urinary gonadotropin excretion in a patient with seminoma of the testis following unilateral orchidectomy

with metastases following removal of a seminoma 11 years previously, in this individual serial observations were conducted over a period of approximately 6 weeks and were in the same range as those in the other subjects

The mean gonadotropin excretion in patients with seminoma was 32 HMG units per 24 hours (Table III) This figure

is considerably higher than that in normal males of comparable age in whom the gonadotropin excretion ranges from 5 to 20 HMG units per 24 hours Typical examples of the excretion patterns in individual subjects are shown in Figs 3 and 4 It should be noted that treatment by abdominal irradiation produced no effect on gonadotropin excretion

In attempting to elucidate the cause of the high gonado tropin excretion it was obviously of importance to determine

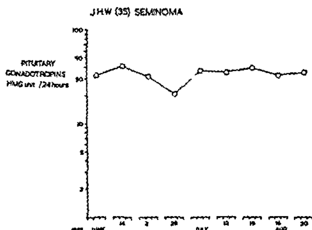


FIG 4 Urinary gonadotropin excretion in a patient with seminoma of the testis following unilateral orchidectomy

whether the operation of unilateral orchidectomy *per se* played any part Accordingly a comparison was made be tween the gonadotropin excretion in patients with seminoma and that in a series of five patients subjected to unilateral orchidectomy for reasons other than seminoma The condi tions apart from seminoma for which unilateral orchidectomy was performed included epididymo orchitis hydrocele and haematocele and the results of the comparison are shown in Table III It will be noted that the difference between the mean values in the seminoma and so called control' groups was highly significant ($P < 0\ 001$) and it can therefore be

Table III

COMPARISON OF URINARY GONADOTROPIN EXCRETION IN SEMINOMA WITH THAT IN PATIENTS SUBJECTED TO UNILATERAL ORCHIDECTOMY FOR REASONS OTHER THAN SEMINOMA

	Number of patients	*Number of observations*	*Mean gonadotropin excretion (HMG units/24 hrs)*	S D *log*	*Range—HMG units/24 hrs (P = 0 95)*
Seminoma	9	63	32	0 22	1[illegible]–8
Unilateral orchidectomy	5	18	12	0 14	1–[illegible]3

Analysis of significance

	t	P
Seminoma *v* Unilateral orchidectomy	6 97	<0 001

concluded that the operation of unilateral orchidectomy *per se* was not responsible for the increased pituitary activity in patients with seminoma

Comment

The results reported in this paper are in general agreement with those of Hamburger and his colleagues (Hamburger and Godtfredsen 1941 Hamburger 1946 1958) who also showed that the gonadotropin excretion in patients with seminoma of the testis is higher than that in normal male subjects Unfortunately Hamburger s results were expressed in mouse uterine units rather than in terms of a standard preparation and for this reason are of limited quantitative significance

The evidence at present available indicates that the anterior pituitary and not the tumour tissue itself or its metastases is responsible for the elaboration of the gonadotropins (Hamburger 1946) The cause of the increased pituitary activity in cases of seminoma is not yet known and the problem remains one for future elucidation

Relationship between urinary gonadotropin and urinary oestrogen excretion during the normal menstrual cycle

The gonadotropin excretion during the normal menstrual cycle has been studied by numerous investigators including Smith and Smith (1936) Klinefelter Albright and Griswold (1943) Pedersen Bjergaard and Tønnesen (1948) Borth Lunenfeld and de Watteville (1957) and Brown Klopper and Loraine (1958) It has been generally found that the output is low in the follicular and luteal phases of the cycle and that a peak of excretion occurs at midcycle coinciding approximately with the time of ovulation However Main and co workers (1943) have reported that in a proportion of patients a second gonadotropin peak may occur just before the onset of menstruation while Heller and co workers (1944) found the pattern of gonadotropin excretion to be very variable from one subject to another

It is probable that the lack of uniformity in the results reported by earlier investigators depends to some extent at least on the unsatisfactory nature of the assay methods used and on the absence of serial estimations in individual patients Recently Brown Klopper and Loraine (1958) using more reliable methods have studied the excretion of oestrogens, pregnanediol and gonadotropins throughout nine ovulatory menstrual cycles and it is probable that the results obtained by these workers are more meaningful from the quantitative point of view than any previously reported

A typical example of the excretion patterns obtained is shown in Fig 5 In this subject parallel assays for gonadotropins were performed on the same urine samples using the mouse uterus and hypophysectomized rat prostate tests At all stages of the cycle the results obtained by the two methods agreed very closely

By performing parallel determinations of urinary oestrogens and gonadotropins in the same patients Brown Klopper and Loraine (1958) were able to investigate the relationship

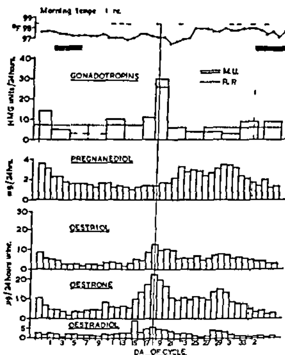


FIG 5 Hormone excretion during a normal menstrual cycle (From Brown Klopper and Loraine 1958 Reproduced by permission of the Editors *J Endocr*)

Gonadotropin assays
(i) —— results by mouse uterus test.
(ii) - - - - results by mouse uterus test in which the reading is actually less than the value shown
(iii) results by hypophysectomized rat prostate test

The vertical line indicates the day of the midcycle peak of excretion of oestrone and oestradiol 17β
▬ = menstrual period

between the gonadotropin and oestrogen peaks which occur at approximately midcycle and which are presumably associated with ovulation They noted that these two peaks did not

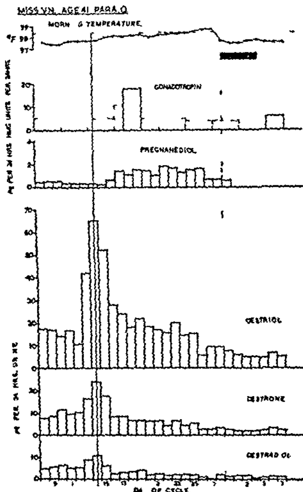


FIG 8 Hormone excretion during a normal menstrual cycle (From Brown Klopper and Loraine 1958 Reproduced by permission of the Editors *J Endocr*)

Gonadotropin assays

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(ii) ——— results by mouse uterus test in which the reading is actually less than the value shown.

The vertical line indicates the day of the midcycle peak of excretion of oestrone and oestradiol 17β

▬ = menstrual period.

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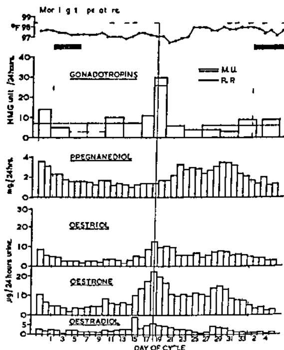


FIG 5 Hormone excretion during a normal menstrual cycle (From Brown Klopper and Loraine 1958 Reproduced by permission of the Editors *J Endocr*)

Gonadotropin assays
(i) —— results by mouse uterus test
(ii) ----- results by mouse uterus test in which the reading is actually less than the value shown
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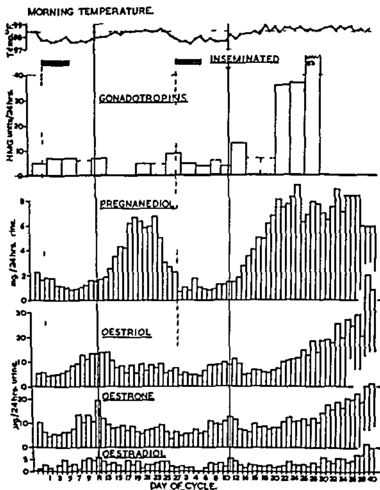


FIG 7 Hormone excretion during a normal menstrual cycle and following a successful artificial insemination (From Brown Klopper and Loraine 1958 Reproduced by permission of the Editors *J Endocr*) Notation as in Fig 6

they contain two gonadotropins which are always present in the same relative proportions

bear a constant relationship in time In some subjects the peaks occurred simultaneously, in others the gonadotropin peak followed the oestrogen peak by periods ranging from 1 to 4 days but in none did the gonadotropin peak precede the oestrogen peak The fact that the gonadotropin peak can occur at some time after the oestrogen peak is well illustrated by the findings in Fig 6 where the two peaks were separated by a period of 4 days

Findings subsequent to artificial insemination

These are illustrated in Fig 7 This subject was artifically inseminated on the day of the cycle on which the peaks of excretion of oestrone and oestradiol 17β were expected to occur Previous inseminations performed at the time of the rise in basal temperature had failed On this occasion the insemination was successful the patient became pregnant and the pregnancy proceeded uneventfully to term The first indication of conception was the sharp rise in gonadotropin excretion nine days after insemination It is probable that this rise was due to the presence in urine of human chorionic gonadotropin (HCG) rather than of pituitary gonadotropins The increase in gonadotropin output was followed by a marked rise in oestrogen excretion and by maintenance of the high luteal phase levels of urinary pregnanediol

Comment

Buchholz (1957) and Brown Klopper and Loraine (1958) have shown that when gonadotropins were assayed in the same urine samples by the mouse uterus and hypophysectomized rat prostate tests the ratio of potencies of the two methods approximated to unity at all stages of the menstrual cycle It might have been anticipated that varying proportions of urinary FSH to urinary ICSH at different stages of the cycle would have been reflected in differences in this potency ratio The fact that the ratio remains relatively constant throughout the cycle suggests either that the extracts contain a single gonadotropin with two activities or that

HAMBURGER C and GODTFREDSEN E (1941) *Acta path microbiol scand* 18 845
HELLER C G FARNEY J P MORGAN P N and MYERS G B (1944) *J clin Endocr* 4 95
KLINEFELTER H F Jr ALBRIGHT F and GRISWOLD G C (1943) *J clin Endocr* 3 529
KLINEFELTER H F Jr REIFENSTEIN E C Jr and ALBRIGHT F (1942) *J clin Endocr* 2 615
KLOPPER A MICHIE E A and BROWN J B (1955) *J Endocr* 12 209
LORAINE J A (1958*a*) *In* Endocrine Aspects of Breast Cancer p 158 ed Currie A R Edinburgh Livingstone
LORAINE J A (1958*b*) The Clinical Application of Hormone Assay Edinburgh Livingstone
LORAINE J A (1959*a*) *In* Cancer vol 6 ed Raven R W London Butterworth
LORAINE J A (1959*b*) *Prob Endokr Gormonoter* 3 95
LORAINE J A and BROWN J B (1956) *J clin Endocr* 16 1180
LORAINE J A and BROWN J B (1959) *J Endocr* 18 77
LORAINE J A. and DICZFALUSY E (1958) *J Endocr* 17 425
LORAINE J A and STRONG J A (1958) *Proc II int Symp Mammary Cancer* p 119 ed Severi L Perugia Division of Cancer Research
LORAINE J A STRONG J A and DOUGLAS M (1957) *Lancet* ii 575
MCARTHUR J W (1952) *Endocrinology* 50 304
MAIN R COX W O'NEAL R and STOECKEL J (1943) *J clin Endocr* 3 331
PEDERSEN BJERGAARD K and TØNNESEN M (1948) *Acta endocr (Kbh)* 1 38
PEDERSEN BJERGAARD K and TØNNESEN M (1951) *Acta endocr (Kbh)* 7 270
SMITH G V and SMITH O W (1936) *New Engl J Med* 215 908
STRONG J A (1958) *In* Endocrine Aspects of Breast Cancer p 141 ed Currie A R Edinburgh Livingstone

DISCUSSION

de la Balze If the peak of oestrogens occurs before the peak of gonadotropins does this mean that the pituitary is not the master gland ?

Loraine Some further information on pituitary ovarian relationship during the menstrual cycle may be obtained by assaying gonadotropins in blood as well as urine My colleague Dr Apostolakis has recently developed a method for the quantitative determination of pituitary gonadotropins in blood and we have applied this technique in a very small number of normally menstruating women

Summary

In patients with recurrent and metastatic mammary carcinoma treated by bilateral adrenalectomy and oophorectomy, the mean gonadotropin excretion before the operation did not differ significantly between the group who subsequently showed a remission of disease and the group who failed to respond to this form of therapy Possible reasons for this finding are discussed

Following adrenalectomy and oophorectomy the mean gonadotropin excretion in the group designated "remission" was significantly higher than that in the group designated "no remission"

The gonadotropin output in patients with seminoma of the testis was significantly higher than that in a group of patients of comparable age subjected to unilateral orchidectomy for reasons other than seminoma

In normally menstruating women the midcycle peak in urinary gonadotropin excretion occurred either simultaneously with or some time after the midcycle oestrogen peak When gonadotropin assays were performed by the mouse uterus and hypophysectomized rat prostate tests the results obtained agreed very closely at all stages of the cycle

REFERENCES

BLACKBURN C M ALBERT A SVEIN H J and UIHLEIN A (1956) *Proc Mayo Clin* 31 649

BORTH R LINDER A and LUNENFELD B (1959) *Acta endocr (Kbh)* 31 192

BORTH R LUNENFELD B and DE WATTEVILLE H (1957) *Fertil and Steril* 8 233

BROWN J B (1955) *Biochem J* 60 185

BROWN J B KLOPPER A and LORAINE J A (1958) *J Endocr* 17 401

BUCHHOLZ R (1957) *Z ges exp Med* 128 219

GADDUM J H (1945) *Nature (Lond)* 156 463

GREEP R O VAN DYKE H B and CHOW B F (1942) *Endocrinology* 30 635

HAMBURGER C (1946) *J Endocr* 5 24P

HAMBURGER C (1958) *Ciba Found Coll Endocr* 12 200 London Churchill

HAMBURGER C and GODTFREDSEN E (1941) *Acta path microbiol scand* 18 845

HELLER C G FARNEY J P MORGAN P N and MYERS G B (1944) *J clin Endocr* 4, 95

KLINEFELTER H F Jr ALBRIGHT F and GRISWOLD G C (1943) *J clin Endocr* 3 529

KLINEFELTER H F Jr REIFENSTEIN E C Jr and ALBRIGHT F (1942) *J clin Endocr* 2 615

KLOPPER A MICHIE E A and BROWN J B (1955) *J Endocr* 12 209

LORAINE J A (1958*a*) *In* Endocrine Aspects of Breast Cancer p 158 ed Currie A R Edinburgh Livingstone

LORAINE J A (1958*b*) The Clinical Application of Hormone Assay Edinburgh Livingstone

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LORAINE J A (1959*b*) *Prob Endokr Gormonoter* 3 35

LORAINE J A and BROWN J B (1956) *J clin Endocr* 16 1180

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LORAINE J A and STRONG J A (1958) *Proc II int Symp Mammary Cancer* p 119 ed Severi L Perugia Division of Cancer Research

LORAINE J A STRONG J A and DOUGLAS M (1957) *Lancet* ii 575

MCARTHUR J W (1952) *Endocrinology* 50 304

MAIN R COX W O NEAL R and STOECKEL J (1943) *J clin Endocr* 3 331

PEDERSEN BJERGAARD K and TØNNESEN M (1948) *Acta endocr (Kbh)* 1 38

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DISCUSSION

de la Balze If the peak of oestrogens occurs before the peak of gonadotropins does this mean that the pituitary is not the master gland ?

Loraine Some further information on pituitary ovarian relationship during the menstrual cycle may be obtained by assaying gonadotropins in blood as well as urine My colleague Dr Apostolakis has recently developed a method for the quantitative determination of pituitary gonadotropins in blood and we have applied this technique in a very small number of normally menstruating women

(Apostolakis M (1960) *J Endocr* in press) In a proportion of subjects we were able to demonstrate gonadotropic activity at approximately midcycle but in no case was activity detected in the follicular or luteal phase of the cycle

To postulate 'ovarian autonomy' during the cycle is certainly a stimulating hypothesis! At the moment I should emphasize that it is only a hypothesis

Li What hormone is responsible for the uterine growth measured by the mouse uterus test? As you have indicated the ventral prostate test in the hypophysectomized rat gives results very similar to those obtained by the uterine test in the mouse We have found indications that both FSH and ICSH are responsible for uterine growth whereas the ventral prostate does not seem to be influenced by the presence of FSH and thus the ventral prostate effect appears to be a very specific index for ICSH (Lostroh A J and Li C H (1956) *Rev argent Endocr* 2 213 Randolph P W Lostroh A J Grattarola R Squire P C and Li C H (1959) *Endocrinology* 65 433)

Loraine I should be unhappy about designating a test 'specific' for one or other of the gonadotropins without the use of hypophysectomized animals The similarity between results obtained by the mouse uterus and hypophysectomized rat prostate tests might be explained by the fact that the two tests measure the same hormone It could also mean that one is measuring two substances which are always present in the same relative proportions We usually term the material determined by the mouse uterus test as 'total gonadotropic activity' this is a convenient term but it tends to mask our ignorance!

Venning Sometimes peaks of gonadotropin excretion are observed during the luteal phase Do you think this can be attributed to variations in clearance of the hormone or to an increased production?

Loraine So far we have not seen peaks of gonadotropin excretion after the fifteenth day of the cycle I do not know whether the gonadotropin peak is in any way related to the renal clearance of the hormone

Venning I noticed also that you used pooled samples of urine do you know how long the peak of excretion of gonadotropins lasts?

Loraine The peak of excretion can last as long as eight days in certain subjects In most individuals the duration is much shorter and in some no peak occurs It seems that young nulliparous individuals have less tendency to show a gonadotropin peak at midcycle than older parous women

Luft Dr Loraine do you think that the general condition of

patients was of any significance for the excretion of gonadotropins? What I mean is this some patients mainly those who responded well to the ablative procedures—and therefore showed an improvement in their general condition—also showed an increase in urinary gonadotropins while those who did not respond to the treatment did not increase the excretion of the same gonadotropins

Loraine I think that the general condition of the patient may be very important We have generally found that in moribund subjects the gonadotropin excretion is very low It is possible that the high gonadotropin levels in patients responding well to adrenalectomy are merely a reflection of their satisfactory general condition

Sonenberg Several years ago Segaloff and co workers reported that prolactin acts synergistically with ICSH in the hypophysectomized ventral prostate test (Segaloff A Steelman S L and Flores A (1956) *Endocrinology* 59 233) Have you any experience with that or with the mouse uterus test?

Loraine Dr Diczfalusy and I have investigated this problem (Diczfalusy E and Loraine J A (1955) *J clin Endocr* 15 424) We were unable to show that prolactin had any synergistic effect on the mouse uterus test rat uterus test and hypophysectomized rat prostate test We concluded that the hypophysectomized rat prostate test was a reasonably specific method for the estimation of urinary ICSH activity

Li With reference to the synergistic problem in the Sprague Dawley strain of rats prolactin is synergistic to ICSH using the ventral prostate weight test of young hypophysectomized animals—I mean 21 day old animals In older animals the synergistic effect is not very evident In the Long Evans strain we cannot find any synergism of prolactin On the other hand growth hormone is extremely good synergistic material with ICSH for ventral prostate of hypophysectomized young rats and young mice

Loraine The amounts of growth hormone present in human urine are probably very small indeed and it may not be important in this particular reaction as regards urine

Gemzell Dr Loraine what do you think about the use of the HMG standard in the Steelman Pohley test for pituitary FSH? We have found quite a good parallelism when testing the partially purified preparations but when we come to the purer preparations the slopes differ considerably Do you think it would be better to use an animal preparation of FSH instead of HMG?

Loraine It might well be better to use an animal FSH rather than HMG We and others have found that with urine extracts it is very important to prepare the urine in the same way as the standard For example one cannot assay urine extracts prepared by the tannic

acid method in terms of a standard such as HMG 20A prepared by the kaolin acetone method

de Ulhoa Cintra Have you compared the alcohol precipitation method with the kaolin method?

Loraine We have not used the alcohol precipitation method because we find the extracts too bulky and too toxic If one attempts to purify such extracts e g by dialysis, a considerable loss of gonado tropic activity occurs

de Ulhoa Cintra What about standardization—by the oestradiol standard injection?

Loraine This is certainly a possibility but it could only be applied to a very limited number of assay methods In addition the validity tests for such an assay might not be satisfactory

Venning Recently Umberger and Gass reported that the pH of the extract affected the biological activity of chorionic gonadotropin (Umberger, E J and Gass G H (1959) *Science* 129 1738) Have you observed this effect with FSH?

Loraine No Our experience with administered FSH has been very little We feel however that for extraction of gonadotropins from urine by the kaolin acetone method control of pH is very important

Wilhelmi Dr Stanley Ellis in our laboratory has been assaying FSH and became quite excited by the report of Umberger and Gass (1959 *loc cit*) He has repeated this observation using the Steelman Pohley test at pH 4 pH 7 and pH 9 Neither the human chorionic gonadotropin base nor the FSH response was different at any one of these pHs The only difference in these observations was that in the report in *Science* the solutions were all buffered and he merely adjusted the pH without buffering so if there is an effect it may be attributed to the buffer which is used Would you agree Prof Li?

Li Yes I agree with you

Gemzell The pure preparation of FSH is very unstable we have found that in a solution at pH 7 the activity dropped to about 10 per cent after 7–10 days

Li In our experience it is quite stable

Gemzell All our preparations have been stable except the last one which was only 0 5 per cent of the starting material

Li Dr Loraine would you comment on the so called FSH ICSH complex with HMG Do you believe that HMG is a dual or a separate hormone?

Loraine All we can say is that with the kaolin acetone method of extraction results obtained by the mouse uterus and hypophysectomized rat prostate tests agree It is possible that by this method we are preparing one substance with two activities I do not know

whether such a conclusion would be justified with other extraction methods such as those depending on benzoic acid adsorption or tannic acid precipitation

Read Dr Loraine would you care to speculate on why gonadotropins should go up after adrenalectomy?

Loraine The cause of the differing excretion patterns following adrenalectomy is at present obscure and further work is necessary before any conclusion can be drawn One possible explanation might be that the high gonadotropin excretion in the group of patients designated remission is a relatively non specific effect occurring in association with an improvement in the general condition of the patient It is also possible that in patients who fail to respond to adrenalectomy the body continues to produce an as yet unknown substance possibly an oestrogen which has an adverse effect on the course of the disease and also acts as a pituitary inhibitor In patients who respond favourably to adrenalectomy on the other hand the production of this hypothetical substance might be decreased or temporarily abolished and for this reason the gonadotropin excretion would tend to rise and would remain high during the period of remission of disease It is conceivable that by administering gonadotropins to patients showing a low excretion following adrenalectomy some therapeutic benefit might ensue

Pearson Concerning Dr Loraine s remark about the possible beneficial effects of gonadotropins we have studied the gonadotropin excretory levels in a group of mammary cancer patients prior to hypophysectomy We found as he did that they do not vary between patients who respond and those who do not respond of course after hypophysectomy they invariably disappear The results of hypophysectomy in the treatment of breast cancer are as good as and perhaps better than those of adrenalectomy and oöphorectomy so that this observation seems to vindicate the gonadotropin aspect

Loraine I agree with that

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the Leydig cells because when the hormone is injected into castrated animals no reaction can be obtained in the seminal vesicles and prostate Therefore extracts to be assayed for ICSH must not contain androgens or oestrogens which act directly on these end organs

McArthur in 1952 using the kaolin method of Bradbury, Brown and Brown (1949) detected the presence of ICSH in the urine of normal women using hypophysectomized immature male rats as the test animals The rats were injected twice daily during a 4 day period and on the fifth day the weight of the prostate and seminal vesicles was determined Since 1953 we have been making use of a test developed by us (Morató Manaro, 1953) for the determination of ICSH in urine This method depends upon the increase in the number of mitoses within the seminal vesicles of the immature rat following injection of ICSH and treatment with colchicine In our opinion this test possesses some practical advantages over the former method while retaining its specificity In using normal immature rats we assume that their testes are in a resting condition i e that they are not secreting androgens If the testes or the adrenal cortex are in fact secreting androgenic hormones the rate must be exceedingly low since in the majority of control animals it is exceptional to find more than 4–5 mitoses per field in tissue sections examined at a magnification of 500 ×

Method and material

Urine extraction is carried out by the method of Bradbury Brown and Brown (1949) with the addition of a final purification step The final extract as obtained by the Bradbury method is not completely water soluble and may give rise to considerable local reaction with oedema Injection of a 24 hour equivalent of urine occasionally results in death of the animals and in diminished sensitivity Aliquots equivalent to 12 hours of urine when injected without purification frequently give rise to a negative reaction while after purification other aliquots of the same extract give rise to uniformly

A METHOD OF DETERMINING INTERSTITIAL CELL STIMULATING HORMONE IN URINE SOME RESULTS IN NORMAL AND PATHOLOGICAL CASES

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J MAGGIOLO

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SINCE Zondek s (1935) pioneer work, a great deal of effort has been devoted to studies concerning the excretion of gonadotropic hormones in the urine of non pregnant women Gonadotropic hormones have been shown to be present under many physiological and pathological conditions and much has been learned about the significance of the quantitative variations observed (see Morató Manaro 1940*a*) It has been shown that the pituitary secretes two different gonadotropic hormones the follicle stimulating hormone (FSH) which in male test animals acts on the seminiferous tubules and the interstitial cell stimulating hormone (ICSH) which acts on the Leydig cells (Fraenkel Conrat *et al* 1941)

The presence of ICSH in the urine has proved difficult to demonstrate and conflicting results have been obtained (Tyndale 1939 Evans and Gorbman, 1942 Varney, Kenyon and Koch 1942, Seegar Jones and Bucher 1943) However the development of the kaolin adsorption method has made it possible to prepare urine extracts with high gonadotropic activity and of low toxicity to the experimental animals According to present knowledge ICSH stimulates the Leydig cells of the testis to secrete steroids (androgens and perhaps oestrogens) which, in turn act on the seminal vesicles and the prostate to produce hypertrophy and cellular hyperplasia by mitotic division The ICSH presumably acts directly upon

500 diameters The reaction is considered positive when there are at least twice as many colchicine arrested mitoses in the seminal vesicles of the test animals (average of 10 fields) as are found in control rats of the same litter injected only with colchicine From the injection of the extract until the micro scopic examination the minimal time elapsed is 54 hours In border line cases an additional assay is performed

Advantages and disadvantages of the method

The use of hypophysectomized rats is advantageous from a scientific point of view However it is impractical in many countries where such animals cannot routinely be obtained With the seminal vesicle colchicine method a high degree of correspondence has been obtained between the results of the test and the clinical diagnosis This suggests that there is little likelihood of interference from the animal s own pitui tary Our method is practical inexpensive easy to carry out and rapid, requiring only 54 hours to obtain a result It is objective since various observers obtain very similar results, and the slides may be kept for later examination Its sensi tivity is comparable to that obtained by C H Li with purified pituitary ICSH in hypophysectomized rats A minor dis advantage is the necessity of killing the rats at night How ever we have not been able to devise an injection schedule which is more convenient and at the same time retains maxi mal sensitivity

A method of determination of ICSH in urine could probably be easily based on our investigations (Morató Manaro Hill and Mussio Fournier 1954) and those of Taymor McArthur and Ingersoll (1953) on the effect of ICSH upon the ^{32}P uptake in the rat prostate and seminal vesicles Such a test should be more satisfactory than the one reported in the present paper and while being equally specific it should eliminate the dis advantage of killing the rats at night The histological pro cedure and the time consuming microscopic observation could be eliminated in this way and the whole test could be carried out in a minimum of 32 hours

positive reactions Moreover, after purification, the extract is completely soluble in the volume of water used for injection and the test animals appear to react in a manner similar to those treated with preparations of ICSH and human chorionic gonadotropin (HCG)

The final purification is accomplished by twice extracting the precipitate obtained by the Bradbury method with 2 ml of distilled water The material is centrifuged at 3 000 *g* after each extraction and the supernatants are combined The gonadotropic hormones are precipitated in a centrifuge tube with 10 volumes of acetone the pH being adjusted to 5 5 if necessary The tube is left in the refrigerator for 24 hours and after centrifugation at 3 000 *g* the acetone is discarded The precipitate, which contains the purified ICSH and FSH is dried in a vacuum desiccator and remains stable for at least a month

Male litter mate rats 21–23 days old and weighing 35–42 g are used as test animals The urine extract is dissolved in distilled water in a volume which permits the injection of equivalents of 4 6 8 or 12 hours of urine in approximately 0 5 ml For convenience we prefer to inject the test material between 8 and 9 a m Twenty seven hours later each rat is injected with a freshly prepared solution of colchicine (Merck) in distilled water containing 1–1 5 μg colchicine per g body weight in a volume of approximately 0 5 ml When injected in the correct dose the colchicine has the property of arresting mitosis in metaphase Nine hours after the injection of colchicine the animals are killed and the seminal vesicles excised and fixed in Bouin solution overnight The standard techniques for dehydration and paraffin embedding are followed but the time ordinarily employed for each step is shortened so as to have the blocks ready approximately 4 hours later The sections 5 μ in thickness are stained with iron haematoxylin and mounted in balsam

We try to select fields where there is the greatest number of mitoses The colchicine arrested mitoses of at least ten of these fields are counted at a magnification of approximately

Table I

EFFECTS OF PURE ICSH AND HMG 20A ON THE NUMBER OF MITOSES IN THE SEMINAL VESICLES OF COLCHICINE TREATED IMMATURE RATS

No of animals	*Amount injected*	*Mitoses test/control* — *Average per field*	*Range*	*Per cent positive*
		ICSH		
	mg			
28	0 1	10 12	0 82–46 25	89 2
4	0 08	4 03	2 23– 7 45	100
3	0 075	10 40	3 5[illegible]–18 16	100
3	0 05	2 80	0 64– 4 59	33 3
9	0	—	—	—
		HMG 20A		
	i u			
21	0 5	9 0	0 32–45 5	76 2
16	0 75	19 55	2 64–57 41	100
12	1	26 26	12 50–40 80	100
12	0	—	—	—

Table II

URINARY EXCRETION OF ICSH IN PHYSIOLOGICAL CONDITIONS

No of cases	*Age*	*Diagnostic*	*Reaction of ICSH (with equivalent in hours of urine)*
9	21–28	Normal women	
		ovulatory phase	Pos 6–8 hrs
		postmenstrual phase	Neg 12 hrs
		premenstrual phase	Neg 12 hrs
10	17–54	Normal men 6	Pos 10–12 hrs
		4	Pos 9 hrs
8	7–9	Normal children before puberty 3 ♂ 5 ♀	Neg 24 hrs

Results

(1) We have carried out more than 35 determinations with an ICSH preparation from sheep pituitary kindly supplied by Prof C H Li The minimum dose that produces a positive reaction is similar to that administered by Prof Li to hypophysectomized rats i e about 0 08 mg We have carried out more than 45 determinations using the International Standard Chorionic Gonadotropin (HMG 20A) showing that an amount equal to 0 5 I U produces a positive reaction

(2) In normal regularly menstruating women we have found a peak of ICSH elimination at about the thirteenth day (from 14 to 10 days) before the next menstruation Our test always gave a positive reaction in the ovulatory phase with an amount of extract equivalent to 6 or 8 hours of urine The duration of the menstrual cycle in the women studied varied between 24 and 33 days In the premenstrual and postmenstrual phases the test gave negative results with an extract equivalent to 12 hours of urine In normal men the test was positive with an extract equivalent to 9–12 hours of urine In prepubertal boys and girls the results were negative with an extract equivalent to 24 hours of urine In a normal 8 year old girl in whom four determinations were made one positive result was obtained with an extract equivalent to 24 hours of urine The foregoing results are summarized in Tables I and II

(3) Some pathological cases are presented in Table III

(*a*) Cushing s syndrome with amenorrhoea a 51 year old patient with this syndrome due to adrenal hyperplasia (Morato Manaro Hill and Mussio Fournier 1954) experienced the menopause 2 years later and two patients had adrenal tumours

(*b*) Two patients with the Stein Leventhal syndrome (Cerviño *et al* 1956) after wedge resection of the ovaries both menstruated and eliminated pregnanediol

(*c*) Eight cases of gonadal dysgenesis five corroborated by laparotomoy the elimination of ICSH was consistently high in seven cases in only one did the pituitary fail to show an increase in functional activity (Morató Manaro 1960)

Table III—*cont*

URINARY EXCRETION OF ICSH IN PATHOLOGICAL CONDITIONS—*cont*

MALE

No of cases	*Age*	*Diagnosis*	*Reaction of ICSH* (with equivalent in hours of urine)*
(i) 23	12–34	(i) 12 cases hypogonadism with low ICSH excretion 6 biopsied	3 Pos 14 hrs Neg 12 hrs 1 Pos 20 hrs 5 Neg 12 hrs 1 Neg 14 hrs 1 Neg 16 hrs 1 Neg 19 hrs
	12–41	(ii) 11 cases hypogonadism with normal or high ICSH excretion 3 biopsied	2 Pos 8 hrs 1 Strongly pos 10 hrs 4 Pos 12 hrs 4 Strongly pos 12 hrs
(j) 5	19–35	Klinefelter syndrome 4 biopsied	3 Pos 9 hrs 1 Pos 12 hrs 1 Pos 16 hrs
(k) 1	17	Traumatism of testes Biopsy showed atrophy and fibrosis in one testis	Pos 12 hrs
(l) 2	60 and 63	Male climacteric with epistaxis	1 Pos 7 hrs 1 Pos 8 hrs
(m) 1	68	Castrated 7 months before test	Pos 2 hrs
(n) 1	55	Posthypophysectomy	Strongly pos 24 hrs
		CHILDREN (MALE AND FEMALE)	
(o) 11	3–10	Children with advanced puberty 1 male 10 female	1 Pos 16 hrs 10 Pos 24 hrs

Som f the positiv r ti s might b obtained with an extract equivalent to a lower mbe f hours f urine, but thi was ot investig t d In th r cases n g tive results wer obtained when amounts (quival nts in h ur) below the positive r ction amounts indicated in th table w re a sayed

(*d*) Three cases of secondary amenorrhoea, one postpartum for the past 7 years

(*e*) Eleven patients with three different types of hirsutism two cases in addition to hirsutism had primary amenorrhoea

Table III

URINARY EXCRETION OF ICSH IN PATHOLOGICAL CONDITIONS

FEMALE

No of cases	Age	Diagnostic	Reaction of ICSH* (with equivalent in hours of urine)
(a) 3	21–51	Cushing s syndrome (i) adrenal hyperplasia (ii) adrenal tumours	 Pos 4 hrs Neg 12 hrs
(b) 2	20 and 24	Stein Leventhal syndrome after wedge resection in the ovulatory phase	Pos 12 hrs Pos 9 hrs
(c) 8	14–32	Gonadal dysgenesis with primary amenorrhoea	2 Pos 8 hrs 3 Pos 9 hrs 2 Pos 12 hrs 1 Pos 16 hrs
(d) 3	20–37	Secondary amenorrhoea	1 Pos 12 hrs 1 Pos 14 hrs 1 Neg 12 hrs
(e) 11	14 and 15	(i) 2 cases hirsutism with primary amenorrhoea	1 Pos 9 hrs 1 Pos 12 hrs
	17–28	(ii) 4 cases hirsutism with secondary amenorrhoea 1 with adrenal hyperplasia	1 Pos 9 hrs 1 Pos 12 hrs 1 Pos 16 hrs 1 Neg 16 hrs
	19–36	(iii) 5 cases hirsutism in normally menstruating women (determinations 9–14 days before menstruation)	1 Pos 7 hrs 1 Pos 8 hrs 2 Pos 9 hrs 1 Pos 14 hrs
(f) 1	29	Hydatidiform mole	Pos 0 012 hrs
(g) 1	19	Pseudohermaphrodite	Pos 7 hrs
(h) 1	29	Adrenogenital syndrome adrenal adenoma	Pos 10 hrs

Some of the positive reactions might be obtained with an e tract equivalent to a lower number of hours of urine, but this w not investigated In other cases neg tive results were obtain d when amounts (equival nts in hours) below the po itive reaction mounts indicated in the table were assayed

Discussion and comments

Like all biological tests the one described here can be better appraised when the number of animals used is larger In practice however the number of animals used must be restricted for obvious reasons

In previous studies we have found that from a threshold to a maximal level of stimulation the number of colchicine arrested mitoses in the epithelium of the seminal vesicles produced by androgens is related to the androgenic potency of the preparations used (Morató Manaro 1940*b*) Table I shows that the number of mitoses is also proportional to the quantity of ICSH or HCG administered Thus one can roughly estimate the strength of an extract of urine even with a smaller number of determinations thereby making possible the use of this test for essentially practical purposes

The question arises whether other hormones of pituitary origin might give rise to a positive reaction in the seminal vesicles We have studied this problem and the positive reactions that we have obtained following the injection of large quantities of prolactin (2 5 mg) thyrotropic hormone (TSH) (2 I U) and adrenocorticotropic hormone (ACTH) (12–20 I U) are in all likelihood due to contamination with small amounts of ICSH (Morató Manaro 1960)

Although the number of normal men and women studied is not large it would appear that the level of ICSH excretion in men is higher than that in women except at midcycle The finding of a peak level of ICSH excretion on about the thirteenth day before menstruation is in agreement with the findings of McArthur (1952) Most of the 48 determinations were made during the ovulatory phase urinary pregnanediol estimation (Morató Manaro and Spremolla 1955) being used as a control Of the pathological conditions studied castration was associated with the highest level of ICSH excretion

Summary

A simplified technique for determining the interstitial cell stimulating hormone (ICSH) content of human urine is

in the second type the hirsutism appeared simultaneously or following secondary amenorrhoea (four cases), the remaining five cases were regularly menstruating women (Morato Manaro 1960)

(f) In one case of hydatidiform mole the test was carried out 8 days after evacuation

(g) One pseudohermaphrodite, with male chromosomal sex, eliminated 19 mg of pregnanediol and 19 mg of 17 ketosteroids purified with Girard s Reagent T On operation he was found to have two testes (Morató Manaro 1960)

(h) One patient with adrenogenital syndrome due to an adrenal adenoma she had periods of amenorrhoea and eliminated 457 mg of 17 ketosteroids (purified with Girard s reagent T) (Morató Manaro, 1960)

(i) Twenty three males with hypogonadism 12 presented a low elimination of ICSH in six of these cases biopsy showed immature testes, no spermatozoa and very slight develop ment of interstitial cells and one case presented a high FSH and very low ICSH excretion The remaining eleven had a normal or high ICSH elimination in the urine in three of these biopsy showed different lesions immature testes sclero sis Sertoli cells only and large quantities of Leydig cells (Morato Manaro 1960)

(j) Five patients with Klinefelter s syndrome all corrobor ated by biopsy except one which presented female chromo somal sex

(k) One case with trauma of testes (3 years before testing) biopsy showed atrophy and fibrosis in one testis

(l) Two patients with male climacteric and epistaxis (Morato Manaro *et al* 1956)

(m) One castrated male test carried out 7 months after castration (Morato Manaro *et al* 1956)

(n) One case of pituitary tumour test carried out 2 months after hypophysectomy

(o) Eleven children with advanced puberty of different aetiologies (Morato Manaro *et al* 1958 Navarro *et al* 1957)

VARNEY R F KENYON A T and KOCH F C (1942) *J clin Endocr* 2 137

ZONDEK B (1935) Las Hormonas del Ovario y del Lóbulo Anterior de la Hipófisis Madrid Labor

DISCUSSION

Loraine Dr Morato Manaro have you made any parallel determinations using this method of colchicine mitosis with the ventral prostate test in hypophysectomized animals? This might be helpful in the determination of specificity

Morató Manaro We have not done that because in our country it is very difficult to get hypophysectomized animals

Wilhelmi Prof Young has very kindly allowed me to make a statement about a programme which is in being in the United States in which many of you may be interested and about which many of you may not have heard Since 1956 the Endocrinology Study Section of the National Institutes of Health has had a programme in being for the preparation and distribution of highly purified anterior pituitary hormones except for ACTH to interested investigators both in the United States and abroad We have now distributed considerable amounts of purified bovine growth hormone and of prolactin In the course of the past two years at Emory University Dr Ellis and I have been operating a small pilot plant for the preparation of further amounts of the other hormones We have at the moment available for distribution a purified preparation of sheep prolactin and a purified preparation of sheep FSH The prolactin is available in 25 mg vials as a sterile preparation and the FSH is available in two forms one a small 5 mg vial which we have labelled as a standard which is appropriate for use in an assay and a larger vial of 25 mg which is useful for experimental work From October 1959 on we shall have also available for distribution a small amount of sheep ICSH—luteinizing hormone (LH)—and also a new lot of bovine growth hormone which have been made at Emory University These preparations have been made according to rather rigid standards of purification with special regard to eliminating contaminants so that the primary activity as stated is the major activity of the preparation and there are nearly negligible contaminations with other activities Those who are interested in obtaining material of this kind are invited to write to Dr R T Hill Secretary, Endocrinology Study Section National Institutes of Health Bethesda 14 Md U S A and we would be happy to entertain requests Letters should contain a brief description of the work which is intended and an estimate if it is possible to make one of the

described The method makes use of the increase in the number of mitoses within the seminal vesicles of the immature rat which follows treatment with ICSH or human chorionic gonadotropin (HCG) and colchicine Preliminary values obtained in normal adults and children of both sexes and in patients with various pathological conditions are presented

REFERENCES

BRADBURY J T BROWN E S and BROWN W E (1949) *Proc Soc exp Biol (N Y)* 71 228

CERVIÑO J M RAVERA J J GROSSO O F POU DE SANTIAGO A MORATÓ MANARO J and MUSSIO FOURNIER J C (1956) *Ann Endocr (Paris)* 17 355

EVANS H M and GORBMAN A (1942) *Proc Soc exp Biol (N Y)* 49 674

FRAENKEL CONRAT H LI C H SIMPSON M E and EVANS H M (1941) *Proc Soc exp Biol (N Y)* 48 723

MCARTHUR J W (1952) *Endocrinology* 50 304

MCARTHUR J W INGERSOLL F M and WORCESTER J (1958) *J clin Endocr* 18 460 1186 1202

MORATÓ MANARO J (1940*a*) *Arch urug Med* 16 219

MORATÓ MANARO J (1940*b*) *Arch clin Inst Endocr (Montevideo)* 1 357

MORATÓ MANARO J (1953) *An Fac Med Montevideo* 38 455

MORATÓ MANARO J (1960) To be published

MORATÓ MANARO J CERVIÑO J M MAGGIOLO J NAVARRO A and POLLAK E (1958) *Rev argent Endocr* 4 1

MORATÓ MANARO J CERVIÑO J M MUSSIO FOURNIER J C POLLAK E and Navarro A (1954) *Bull Soc Méd Paris* 70 517

MORATÓ MANARO J HILL W S and MUSSIO FOURNIER J C (1954) *Presse méd* 62 1537

MORATÓ MANARO J MAGGIOLO J ROCCA F and CERVIÑO J M (1956) *An Fac Med Montevideo* 41 191

MORATÓ MANARO J and SPREMOLLA F (1955) *An Fac Med Montevideo* 40 260

NAVARRO A ZALDUA DE DELFINO E MORATÓ MANARO J GROSSO O F CERVINO J M MAGGIOLO J and MUXI F (1957) *Abstr II Congr PanAmer Endocr* 1 303

SEEGAR JONES G E and BUCHER N L R (1943) *Endocrinology* 32 46

TAYMOR M L MCARTHUR J W and INGERSOLL F M (1953) *Surg Forum* 52 317

TYNDALE H H (1939) Quoted by Engle E T (1939) Sex and Internal Secretions p 1003 Baltimore Williams & Wilkins

ADRENOCORTICOTROPIC HORMONE AND MELANOCYTE-STIMULATING HORMONE FROM HUMAN PITUITARY GLANDS

TEH H LEE AARON B LERNER AND
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THE physiological importance of adrenocorticotropic hormone (ACTH) has stimulated research interests for many years However, attempts to isolate this hormone and unravel its chemical structure were not undertaken until the introduction of such modern research techniques as ultra centrifugation electrophoresis chromatography counter current distribution *etc* Bell (1954) reported the isolation and complete structure of β corticotropin from porcine pituitary glands Li and co workers elucidated the structure of ovine and bovine corticotropin (Li *et al* 1955 Li Dixon and Chung 1958) It is interesting to note that all the corticotropins are made up of 39 amino acids and that a difference occurs only in the region between amino acid residues 25 and 33 These facts are consistent with the findings of Bell and co workers (1956) that the first 24 or 25 amino acid residues are essential for full biological activity

The isolation of melanocyte stimulating hormones (MSH) from pituitary glands of domestic animals was reported during the past five years Lee and Lerner (1956) showed that two substances possessing melanocyte stimulating hormone activity were present in porcine pituitary glands They proposed that these substances be called α and β MSH The isolation of α MSH had been reported earlier by Lerner and Lee (1955) and that of β MSH by Porath and co workers (1955) and by

amount of hormone that might be required At the moment because our supply of FSH is small we are limiting the amount given to a single individual at a single time—you may ask for it again—to 250 mg but when the growth hormone becomes available there will be no practical limit up to 10 g in the amount that may be asked for For the LH we shall probably place a limit of 100 mg We would be very happy to receive requests from any of you So far in the time that the programme has been in being since November 1956 we have distributed material to nearly 350 investigators in 19 different countries and we would like to extend this programme if possible to make these benefits more widely available for those of you who are seriously interested in working with the endocrine principles of the anterior pituitary

was purified further on a CM cellulose column which had been equilibrated previously with 0 005 M ammonium acetate solution at pH 5 9 The chromatographic column was developed by step wise elution with 0 05 M pH 5 8 and 0 25 M, pH 6 9 ammonium acetate solutions Two active fractions were resolved as shown in Fig 2 Fraction A which

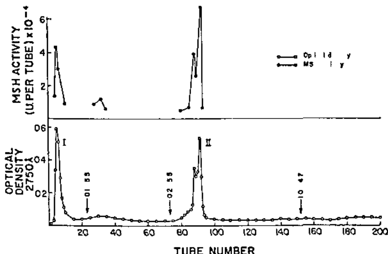


FIG 1 A crude MSH and ACTH concentrate from human pituitary glands was chromatographed on a DEAE cellulose column Gradient elution with 0 1 M and 0 2 M ammonium acetate buffers at pH 5 5 was used and the effluent was collected in 3 ml fractions Arrows indicate the place at which a buffer was added

constituted 1–2 per cent of the original crude concentrate possessed only MSH activity It gave four major peptide fragments on chymotryptic digestion at an enzyme/substrate ratio of 1 100 for 3 hours at 30° and pH 8 3 Three of the fragments behaved similarly to those produced from porcine β MSH during ionophoresis in pyridine acetate buffer at pH 6 5 while the fourth one showed a much lower mobility toward the anode Dixon (1960) succeeded in isolating a melanocyte stimulating hormone from human pituitary

Geschwind and Li (1957) Harris and Roos (1956) and Geschwind, Li and Barnafi (1957*a*) independently found an identical structure for porcine β MSH Subsequently the isolation and elucidation of the structure of bovine β MSH were achieved by Geschwind, Li and Barnafi (1957*b*) The structure of porcine α MSH was reported by Harris and Lerner (1957) and Harris (1959*a*) α MSH also was isolated from bovine pituitary glands Both kinds of β MSH were found in ovine material Bovine and porcine β MSH differ at position 2 where a seryl residue replaces a glutamyl residue However, α MSH from both species is identical

Investigators wanted to find out whether or not human beings in whom these hormones are found effective also secrete the same hormones The present communication is concerned with purification and characterization of MSH and ACTH from human pituitary glands The preliminary steps of the fractionation procedure used were essentially those of Payne Raben and Astwood (1950) in which the active materials were extracted by glacial acetic acid and precipitated by addition of two volumes of petroleum ether The active precipitates were purified further by the oxycellulose adsorption method proposed by Astwood and co workers (1951) The resulting fraction was used as the starting material for ion exchange column chromatography Diethylaminoethyl (DEAE) cellulose and carboxymethyl (CM) cellulose, prepared according to the procedure of Peterson and Sober (1956) were used as the ion exchangers The crude concentrate was adsorbed on a DEAE cellulose column which previously was equilibrated with 0 005 M ammonium acetate solution at pH 6 9 Removal of the adsorbed materials was achieved by gradient elution to pH 5 5 and 0 2 M ammonium acetate concentration Since it is well established that ACTH possesses intrinsic MSH activity, the *in vitro* frog skin bioassay for MSH described by Shizume Lerner and Fitzpatrick (1954) could be used to locate both the MSH and ACTH fractions By means of a DEAE cellulose column two active fractions I and II were obtained as shown in Fig 1 The major fraction, II

phan was found in the intact hormone by the method of Lerner and Barnum (1946) Therefore human corticotropin appeared to have an amino acid composition identical with that of ovine and bovine corticotropin but different from that of porcine β corticotropin in that one leucyl residue was re placed by a seryl group Fraction B was judged homogeneous by the fact that nearly integral molar ratios were obtained upon amino acid analysis Its homogeneity was confirmed further by finding that upon tryptic digestion the number of peptide fragments containing tryptophan histidine methion ine, tyrosine and arginine was consistent with the amino acid composition and the known specificity of the enzyme

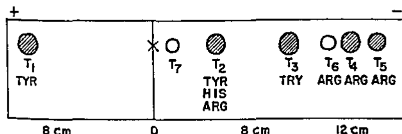


FIG 3 A tryptic digest of human ACTH was subjected to ionophoresis Lined circles indicates spots of high intensity following staining with ninhydrin reagent Amino acids given under the spots are those revealed by specific colour reagents

Paper strip modification of the Edman method carried out on the intact hormone showed the N terminal sequence to be Ser Tyr Ser Met Glu A 24 hour carboxypeptidase digestion of the intact hormone as well as of the C terminal peptide isolated from tryptic digestion gave almost equimolar amounts of phenylalanine glutamic acid and leucine How ever digestion at various durations indicated that the C terminal sequence probably was Leu Glu Phe Tryptic digestion at an enzyme/substrate ratio of 1 100 pH 8 3 3 5 hours at 37 split the intact hormone into four major and three minor fragments They were separated by ionophoresis in pyridine acetate buffer at pH 6 5 as shown in Fig 3 All

material using Zeo Karb 225, 2 per cent cross linked 200–400 mesh as ion exchanger Harris (1959*b*) elucidated its structure and found several additional amino acid residues in its N terminal sequence besides all those present in porcine β MSH It is postulated that fraction A is one of the human MSH's and is identical with that isolated by Dixon Because of the

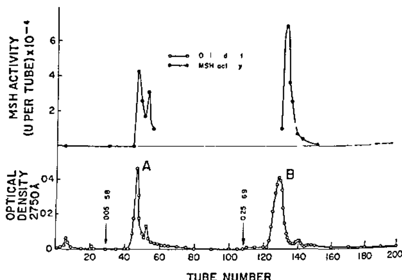


FIG 2 Fraction II prepared from the DEAE cellulose column was chromatographed on a CM cellulose column The latter was developed with 0 05 M and 0 25 M ammonium acetate buffers at pH 5 8 and 6 9 respectively The effluent was collected in 3 ml fractions Arrows indicate the place at which a buffer was added.

lack of sufficient material additional attempts to establish the exact structure of fraction A could not be undertaken at present

Fraction B which constituted 10–30 per cent of the crude concentrate possessed both ACTH and intrinsic MSH activities *An acid hydrolysate of this fraction analysed* by the technique of Moore, Spackman and Stein (1958) gave the following amino acid composition $Ala_3Arg_3Asp_2Glu_5Gly_3$ $His_1Leu_1Lys_4Met_1Phe_3Pro_4Ser_3Tyr\ Val_3$ One mole of trypto

corticotropin When a tryptic digest of human corticotropin was compared with one of porcine corticotropin A (White and Landmann 1955) by ionophoresis a very interesting similarity was observed in the electrophoretic patterns All basic and neutral peptides, T_2 T_3 T_4 T_5 T_6 and T_7 (a mixture) from human corticotropin had the same mobility as their porcine counterparts The only acidic peptide T_1 showed a slightly higher mobility toward the anode This observation seems consistent with the view that peptides T_2 T_3 T_4 T_5 and T_6 of human ACTH might be identical with those derived from other species while T_1, which has the same C terminal sequence

Table I

AMINO ACID COMPOSITION OF PEPTIDE FRAGMENTS FROM TRYPTIC DIGESTION OF HUMAN ACTH

Peptide	*Amino acids*
T_1*	($Asp_2Ser_1Glu_3Pro_3Gly_1Ala_3Val$ Tyr_1Phe_1) Leu† Glu† Phe†
T_2	($Ser_2Tyr_1Met_1Glu_1His_1Phe_1Arg_1$)
T	($Try_1Gly_2Lys_2Pro_1Val_1$)
T_{4a}	($Lys_2Arg_2Pro_1Val_1$)
T_4	($Arg_2Pro_1Val_1Lys_1$)
T	(Lys_1Arg_1)
T	($Arg_2Pro_2Val_1Lys_1$)

* Determined by Moore, Spackman and Stein (1958) column
† Amino acid released by carboxypeptidase

Leu Glu Phe as the intact hormone may possess some difference in amino acid sequence If this were true then peptide T_2 which has a single histidine and methionine residue would occupy the N terminal sequence 1–8 peptide T_3 which has a single tryptophan residue would be at positions 9–15 peptides T_4 T_5 and T_6 would fill various lengths of positions 16–21 and peptide T_1 which has the same C terminal sequence as the intact hormone would occupy the sequence 22–39 (Fig 5) Whether or not this is mere speculation can only be determined by further work which is in progress in this laboratory

As yet we have not examined carefully several minor fractions containing melanocyte stimulating hormone activity

these bands except one, which turned out to be a complex mixture were eluted and the peptides purified by paper chromatography using either the system n butanol acetic acid water (4 1 5) or n butanol acetic acid pyridine water (30 6 24 20) A reproduction of the paper chromatograms is shown in Fig 4 Each peptide was hydrolysed with glass distilled 5 7 N hydrochloric acid in sealed, evacuated

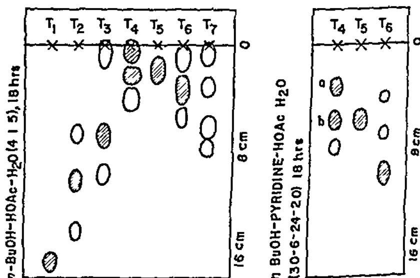


FIG 4 Peptide fragments from a tryptic digest of human ACTH were subjected to paper chromatography Lined circles indicate the spots of high intensity following staining with ninhydrin reagent

tubes at 105° for 20–24 hours The constituent amino acids were identified either by paper chromatography or by the *Moore, Spackman and Stein (1958) column* Table I shows the amino acid composition of the peptides isolated from tryptic digestion

The amino acid sequences of porcine bovine and ovine corticotropins vary only in the region between amino acids 25 and 33 By analogy one may speculate that similar differences might exist in this portion of the structure of human

to determine whether any α MSH like substance is present in human pituitary glands However there is ample evidence to indicate that no sizeable quantity of an α MSH like substance could be isolated from the material available to us In view of the fact that the glands collected at autopsies were usually in very poor condition and were stored in acetone for several months it would not be surprising if large portions of α MSH were lost prior to fractionation

Experimental

Materials Human pituitary glands collected at autopsies were stored in acetone and made into a powder by grinding in a Waring blendor and washing with more cold acetone The powder was dried *in vacuo* and stored at −10° A crude con centrate of human ACTH was also made available to us through the courtesy of Dr M S Raben of the New England Medical Center Boston Mass A highly purified preparation of carboxypeptidase was donated by Dr W F White of Armour and Company Chicago Ill Crystalline trypsin and chymotrypsin were purchased from Mann Research Labora tories New York N Y

Method of MSH assay The *in vitro* bioassay method of Shizume Lerner and Fitzpatrick (1954) using isolated skin of frogs (*Rana pipiens*) was modified to suit our purpose Six teen pieces of skin from a group of four frogs were arranged in a Latin square Two of the four rows of the Latin square were used to assay two unknown solutions while the other two rows were used to construct a dose response curve at two dosage levels using standard hormone solutions The un known solutions were diluted so that their responses were within the range of the dose response curve The changes in skin colour after addition of MSH were measured by a photo electric reflection meter

DEAE cellulose chromatography Preparation of the cellulose and packing of the column followed the procedure of Peterson and Sober (1956) All chromatographic columns

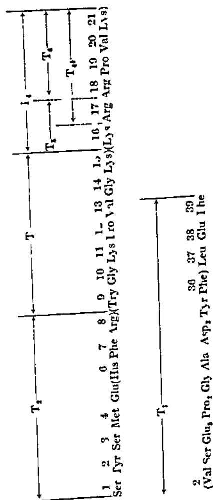


FIG 3 Amino acid sequence of human ACTH

peaks in this case Therefore, step wise introduction of ammonium acetate buffers of increasing molarity was em ployed Two well separated active components were eluted by successive introduction of 0 05 M and 0 25 M ammonium acetate buffers at pH 5 8 and pH 6 9, respectively The operation of the column was stopped after 160 fractions of effluent had been collected The latter were measured for optical density at 2 750 Å wavelength Aliquots of selected fractions were assayed for MSH activity (Fig 2) The active fractions 41–50 and 122–135 were combined separately and lyophilized An aliquot of each lyophilized sample was injected intravenously into hypophysectomized male rats and assayed for ACTH activity by the U S P ascorbic acid depletion test

N and C terminal sequence studies The N terminal sequence of the intact hormone was determined by following the paper strip modification of the Edman phenylthiohy dantoin (PTH) method described by Fraenkel Conrat (1955) Altogether five successive steps of the degradation procedure gave clearcut results The extracted PTH derivatives of N terminal sequence amino acids were measured quantita tively over the range of 2 400–3 000 Å with a Cary model 14 spectrophotometer The yield of PTH amino acid at each step was about 60 per cent The PTH derivatives were identi fied by submitting an aliquot to paper chromatography using an *n* heptane pyridine system (7 3) with known PTH amino acids as reference The PTH compounds except for the serine derivative were hydrolysed with 5 7 N hydrochloric acid in sealed evacuated tubes at 150° for 20 hours To con firm the identity of the PTH derivatives the regenerated amino acids were identified by paper chromatography For C terminal sequence studies the carboxypeptidase method described by Harris (1955) was followed About 2 mg of the ACTH preparation dissolved in 2 ml of 0 1 per cent $NaHCO_3$ was incubated with carboxypeptidase (0 05 mg in 0 1 per cent $NaHCO_3$ solution at pH 8 0–8 5) that had been treated previously with a 50 mole excess of diisopropylfluorophosphate

were run at 5° Three grams of DEAE cellulose were used for a column of 11 × 180 mm The column was equilibrated by prolonged washing with approximately 1,000 ml of 0 005 M ammonium acetate solution at pH 6 9 About 50 mg of a crude MSH and ACTH concentrate was extracted twice with 3 ml portions of the buffer and the clear supernatant solutions were applied to the column The column was developed with 0 005 M ammonium acetate buffer at pH 6 9 introduced through a 250 ml mixing flask which was filled with the same solution and stirred by a magnetic stirrer Sufficient hydrostatic head was applied to the column to maintain a flow rate of 12–15 ml per hour Effluent from the column was collected in 3 ml fractions The unretarded material emerged as a single peak during the first 20 fractions Thereafter, the gradient elution was started by introducing 0 1 M ammonium acetate buffer at pH 5 5 into the mixing flask A small amount of inactive material emerged during the next 50 fractions of effluent Buffer solution of 0 2 M ammonium acetate at pH 5 5 was then introduced to raise the concention gradient The major portion of materials with MSH and ACTH activities emerged as overlapping peaks with this gradient The operation of the column was terminated after a total of 120 fractions had been collected The samples were measured individually for optical density at 2 750 Å wave length Aliquots of selected fractions were assayed for MSH activity after suitable dilution (Fig 1) Fractions 2–10 and fractions 85–95 were combined separately and lyophilized

CM-cellulose chromatography Three grams of dry CM cellulose were packed into a column of 11 × 180 mm The column was equilibrated with 0 005 M ammonium acetate buffer at pH 5 9 The material to be fractionated was extracted twice with 3 ml portions of the starting buffer, and the resultant clear supernatant solutions were applied to the column The column was washed with more of the same buffer to displace the unretarded material The effluent of the column was collected in 3 ml fractions It became evident from pilot runs that gradient elution did not give sharper

peaks in this case Therefore, step wise introduction of ammonium acetate buffers of increasing molarity was employed Two well separated active components were eluted by successive introduction of 0 05 M and 0 25 M ammonium acetate buffers at pH 5 8 and pH 6 9 respectively The operation of the column was stopped after 160 fractions of effluent had been collected The latter were measured for optical density at 2 750 Å wavelength Aliquots of selected fractions were assayed for MSH activity (Fig 2) The active fractions 41–50 and 122–135 were combined separately and lyophilized An aliquot of each lyophilized sample was injected intravenously into hypophysectomized male rats and assayed for ACTH activity by the U S P ascorbic acid depletion test

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At intervals of 0 5, 4 8 and 24 hours 0 5 ml of the reaction mixture was withdrawn and the enzymic reaction was terminated by addition of about 50 mg of Amberlite IR 120 (H^+ form, 20–50 mesh, 8 per cent cross linking) The amino acids released by carboxypeptidase were eluted from the resin by 5 N ammonium hydroxide and identified by unidimensional paper chromatography in either the system *n* butanol acetic acid water (4 1 5) or *m* cresol phenol (1 1) on Whatman No 52 paper buffered by 0 1 M borate at pH 7 3 as described by Levy and Chung (1953)

Tryptic digestion An enzyme/substrate ratio of 1 100 was used for hydrolysis The reaction was carried out in a 10 ml beaker equipped with a pair of glass and calomel electrodes, a microburette and a magnetic stirring bar The temperature of the beaker was maintained at 37° with a water bath Seven milligrams of the ACTH preparation were dissolved in about 2 ml of water and the pH of the solution was adjusted to 8 2 with 0 005 N sodium hydroxide After addition of 0 070 mg trypsin the pH of the solution started to drop The pH was brought back to 8 2 and maintained at that value during the course of digestion (3 5 hours) by manual addition of 0 005 N sodium hydroxide Approximately 3 2 moles of sodium hydroxide were consumed per mole of the hormone The enzymic reaction was terminated by lowering the pH to 2 5–3 0 with 1–2 drops of 0 1 N hydrochloric acid and the reaction mixture was dried over potassium hydroxide pellets in a vacuum desiccator An aliquot of the digest was submitted to ionophoresis in pyridine acetate buffer at pH 6 5 and at a voltage of 25 v/cm in an apparatus described by Michl (1951) The peptide fragments were located on the ionogram by means of ninhydrin reagent Those containing arginine histidine, tryptophan and tyrosine were identified by specific colour reagents (Block Durrum and Zweig 1958) The remainder of the digest was submitted to ionophoresis on Whatman No 3 MM paper with 1 mg digest per cm of paper width and under the optimal conditions found by a pilot run The bands were located by means of

guide strips cut lengthwise from both edges of the ionogram and were eluted separately by 5 per cent acetic acid The eluates were dried over potassium hydroxide *in vacuo* The peptide fragments were purified further by paper chromatography Usually either the system *n* butanol acetic acid water (4 1 5) or *n* butanol acetic acid pyridine water (30 6 24 20) was used The main bands from each paper chromatogram were eluted and dried separately in the usual manner

Chymotryptic digestion An enzyme/substrate ratio of 1 100 was used for the digestion which was carried out in the same manner as those with trypsin except that 0 5 mg of an MSH preparation was used The resultant peptide fragments were not isolated

Quantitative amino acid analysis Hydrolysis of the intact hormone or the isolated peptide fragments was carried out in a sealed evacuated tube with glass double distilled constant boiling hydrochloric acid at 105° for 20–24 hours The hydrolysate was transferred to a small beaker and taken to dryness over potassium hydroxide pellets and phosphorus pentoxide *in vacuo* The dried residue was wetted down once and taken to dryness again in the same manner The amino acid composition of the hydrolysate was determined quantitatively by the Moore Spackman and Stein (1958) technique

Summary

The isolation of adrenocorticotropic hormone (ACTH) and a melanocyte stimulating hormone (MSH) from human pituitary glands is described Human corticotropin has the same amino acid composition as the bovine and ovine hormones but it differs from porcine corticotropin in having one leucyl residue replaced by a seryl residue Its N terminal sequence Ser Tyr Ser Met Glu and C terminal sequence Leu Glu Phe are identical with those of corticotropin from sheep cattle and hogs Preliminary structural studies indicate that the amino acid sequence of human corticotropin is identical with that of corticotropin from other species except in the region between amino acid residues 22–30

Note added in proof The arrangement of the peptide fragments as shown in Fig 5 was confirmed by these additional findings a dipeptide ArgTry was isolated from a chymotryptic digest of the intact hormone and Lys was found to be the C terminus of peptide fragment T_{6}

Acknowledgments

The authors wish to express their gratitude to Dr M S Raben for the crude human ACTH concentrates and to Dr W F White for highly purified carboxypeptidase Dr J D Fisher was most helpful in performing numerous ACTH assays

REFERENCES

ASTWOOD E B RABEN M S PAYNE R W and GRADY A B (1951) *J Amer chem Soc* 73 2969
BELL P H (1954) *J Amer chem Soc* 76 5565
BELL P H HOWARD K S SHEPHERD R G FINN B M and MEISENHELDER J H (1956) *J Amer chem Soc* 78, 5059
BLOCK R J DURRUM E L and ZWEIG G (1958) A Manual of Paper Chromatography and Paper Electrophoresis p 128 New York Academic Press
DIXON H B F (1960) *Biochim biophys Acta* 37 38
FRAENKEL CONRAT H (1955) *In* Methods of Biochemical Analysis p 383 ed Glick D New York Interscience Publishers
GESCHWIND I I and LI C H (1957) *J Amer chem Soc* 79 615
GESCHWIND I I LI C H and BARNAFI L (1957a) *J Amer chem Soc* 79 620
GESCHWIND I I LI C H and BARNAFI L (1957b) *J Amer chem Soc* 79 1003
HARRIS J I (1955) *In* Methods of Biochemical Analysis p 397 ed Glick D New York Interscience Publishers
HARRIS J I (1959a) *Biochem J* 71 451
HARRIS J I (1959b) *Nature (Lond)* 184 167
HARRIS J I and LERNER A B (1957) *Nature (Lond)* 179 1346
HARRIS J I and ROOS P (1956) *Nature (Lond)* 178 90
LEE T H and LERNER A B (1956) *J biol Chem* 221 943
LERNER A B and BARNUM C P (1946) *Arch Biochem* 10 417
LERNER A B and LEE T H (1955) *J Amer chem Soc* 77 1066
LEVY A L and CHUNG D (1953) *Analyt Chem* 25 396
LI C H DIXON J S and CHUNG D (1958) *J Amer chem Soc* 80 2587
LI C H GESCHWIND I I COLE R D RAACKE I D HARRIS J I and DIXON J S (1955) *Nature (Lond)* 176 687
MICHL H (1951) *Mh Chem* 82 489
MOORE S SPACKMAN D H and STEIN W H (1958) *Anal Chem* 30 1185

PAYNE R W RABEN M S and ASTWOOD E B (1950) *J biol Chem* **187** 719
PETERSON E A and SOBER H A (1956) *J Amer chem Soc* **78** 751
PORATH J ROOS P LANDGREBE F W and MITCHELL G M (1955) *Biochim biophys Acta* **17** 598
SHIZUME K LERNER A B and FITZPATRICK T B (1954) *Endocrinology* **54** 553
WHITE W F and LANDMANN W A (1955) *J Amer chem Soc* **77** 1711

[In the absence of Dr Lee this paper was presented by Dr Harris Discussion of this paper was postponed until after the paper by Dr Harris —EDS]

A MELANOCYTE-STIMULATING HORMONE FROM THE HUMAN PITUITARY GLAND

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MELANOCYTE STIMULATING substances from pig and ox pituitary glands have been isolated in pure form and characterized in terms of their chemical structures β Melanocyte stimulating hormone (β MSH) from both species has been shown to be a polypeptide consisting of 18 amino acid residues seven of which occur in the same sequence as was previously shown to occur in the adrenocorticotropic hormone (ACTH) molecule α Melanocyte stimulating hormone (α MSH) on the other hand was found to be the *N* acetyl derivative of a tridecapeptide amide in which the sequence of amino acids is identical with the N terminal tridecapeptide sequence in corticotropin The biological relationships which exist between MSH s and corticotropins are thus reflected in terms of their respective chemical structures (for review, see Harris 1959*a* and *b*)

Extracts of human pituitary glands have long been known to possess melanocyte stimulating activity when assayed in frogs and very recently Dr H B F Dixon at Cambridge has succeeded in isolating a chromatographically pure melanocyte stimulating substance from the human gland This material generously donated by Dr Dixon has been submitted to chemical analysis and experiments which have led to the elucidation of its complete chemical structure are now described It should be stated at the outset that the methods which have been used were to a very large extent imposed by the limited amount (4–5 mg) of the hormone which was available for study

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The structure of human MSH (Harris 1959*b*)

The hormone was found to be a polypeptide containing 13 different amino acids namely alanine arginine aspartic acid, glutamic acid glycine histidine lysine, methionine, phenylalanine proline serine tyrosine and tryptophan The presence of three aromatic amino acids suggested that the molecule could be broken down into peptide fragments of convenient size by means of chymotrypsin A sample of the hormone was allowed to react with the enzyme and as predicted it was hydrolysed in a specific manner to give four major peptide fragments which could be separated by ionophoresis on paper (Fig 1*b*) a fifth relatively minor component

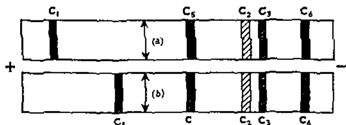


FIG 1 Products of chymotryptic hydrolysis of pig β MSH (*a*) and human β MSH (*b*) Ionophoresis in pyridine acetic acid pH 6 4 40 v/cm 2 hrs

(peptide C_2 Fig 1*b*) was later shown to be an intermediate product The amino acid compositions and C terminal groups of these five peptides are listed in Table I

Table I

AMINO ACID COMPOSITION AND C TERMINAL AMINO ACIDS OF PEPTIDES PRODUCED BY CHYMOTRYPTIC HYDROLYSIS OF HUMAN β MELANOCYTE STIMULATING HORMONE

Peptide	*Amino acid composition*	*C terminal group*
C	Ala Asp Glu Gly Lys Pro Tyr	Tyr
C_3	Arg Asp Gly Lys Pro Ser Try	Asp
C	Arg Glu His Met Phe	Phe
C_4	Asp Gly Lys Pro Ser	Asp
C	Arg Try	Try

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Table II

AMINO ACID COMPOSITION AND C-TERMINAL AMINO ACIDS OF PEPTIDES PRODUCED BY TRYPTIC HYDROLYSIS OF PEPTIDE C_2

Peptide	*Amino acid composition*	*C terminal group*
C_1 TA	Asp Glu Gly Lys Pro Tyr	Tyr
TB_1	Ala Glu Lys	Lys
TA_2	Asp Glu Gly Pro Tyr	Tyr
TB_2	Lys	—

In addition one of the minor components (TA_2) appeared to be identical with the N terminal peptide Asp Glu Gly Pro Tyr (peptide C_1 Fig 1*a*) derived from pig β MSH

Peptide C_2 gave additional amounts of C_5 and C_6 when it was separately redigested with chymotrypsin, showing that it was a product of partial hydrolysis and that C_5 and C_6 occupied adjacent positions in the molecule and since both C_2 and C_5 contained C terminal aspartic acid it was clear that these peptides formed the C terminal sequence Arg Try (Gly,Ser, Pro Lys) Asp in human β MSH

In order to complete the sequence analysis peptides TA_1, TB_1 C_3 and C_5 were selected for further study They were submitted to partial hydrolysis using concentrated hydrochloric acid and the bacterial enzyme subtilisin the results are summarized in Table III

Table III

PEPTIDES IDENTIFIED IN PARTIAL ACID* (12 N HYDROCHLORIC ACID 8") AND SUBTILISIN† HYDROLYSATES

Peptide	*Products of partial hydrolysis*
*C_1TB_1	Ala Glu Glu Lys
C_1TA_1	Lys Asp Lys Asp Glu Glu Gly Gly Pro Tyr
*C_5	Ser Pro Ser Pro Pro Ser Pro Pro Lys Pro Pro Lys Pro Lys Lys Asp
†C_3	Arg Met Arg Met Glu Glu His Glu His Phe

It was not found possible to undertake quantitative amino acid and end group analyses on the parent molecule nevertheless the results (summarized in Tables I–III) obtained with

It was immediately apparent that the melanocyte stimulating substance from the human gland was of the β MSH type and that it was structurally related to pig β MSH Thus peptides C_{α} C_3, C_5 and C_6 could not be distinguished from the corresponding peptide fragments derived from pig β MSH (Fig 1*a*) as judged by electrophoretic and chromatographic behaviour, amino acid composition (with the exception of C_3 which contains arginine instead of lysine) and C terminal group analysis Peptide C_1 on the other hand, differs from the corresponding peptide from pig β MSH both in electrophoretic

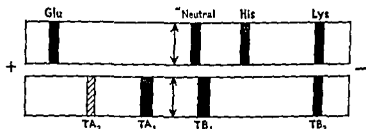


FIG 2 Products of tryptic hydrolysis of peptide C Iono phoresis in pyridine acetic acid pH 6 4 40 v/cm 90 min

and chromatographic behaviour and in amino acid composition although aspartic acid glutamic acid glycine and proline, as well as the C terminal amino acid tyrosine are common to both peptides

The presence of lysine suggested that C_1 would be susceptible to the action of trypsin It was therefore reacted with the enzyme and the products of reaction were separated by ionophoresis on paper as shown in Fig 2 their amino acid compositions and C terminal groups were determined and the results are summarized in Table II

The two major products of reaction, TA_1 and TB_1 were both found *to contain lysine, and the fact that free lysine* (TB) was also released by the enzyme suggested that C_1 contained a Lys Lys bond and that it formed the N terminal sequence (Ala,Glu) Lys Lys (Asp Glu,Gly,Pro) Tyr in human β MSH

related positions in polypeptides isolated from different species and involving the replacement of one amino acid by another which is chemically related to it have been encountered pre viously among for example vasopressins hypertensins in sulins and corticotropins the occurrence of the additional tetrapeptide in human MSH however represents an unpre cedented structural species variation among naturally occur ring biologically active polypeptide and protein molecules of relatively low molecular weight For example all known insulins (Harris Sanger and Naughton 1956) and cortico tropins including human corticotropin (see Lee Lerner and Buettner Janusch this colloquium p 251 cf Li 1958) con tain 51 and 39 amino acids respectively growth hormones from different species on the other hand appear to show considerable variations in molecular size (Li 1958)

Pig and ox β MSH s differ only in position 2 (Fig 3) and in this case a glutamic acid residue in the pig hormone is replaced by the chemically *unrelated* amino acid serine in the ox hor mone It is perhaps significant that a replacement of this type has occurred in a part of the molecule which does not appear to be essential for biological activity A similar situa tion exists among corticotropins where structural changes of this type are confined to the C terminal segment (positions 25–39) of the molecule which is known not to be essential for biological activity On the other hand the N terminal sequence of 24 amino acids which forms the smallest known active fragment is identical in all corticotropins so far studied

It seems likely therefore that genetical alterations which cause structural changes involving the replacement of an amino acid by another which is chemically unrelated to it in essential parts of a molecule would result in the formation of molecules of altered biological specificity Thus in the case of haemoglobins for example the replacement of a glutamic acid residue in normal haemoglobin by a valine residue leads to the formation of the abnormal sickle cell haemoglobin (Ingram 1957)

peptide fragments derived from it by hydrolysis with chymo trypsin and trypsin appear to show quite conclusively that human melanocyte stimulating hormone is of the β MSH type, and that it is a polypeptide containing twenty two amino acids which occur in the following sequence

1	2	3	4	5	6	7	8	9	10	11	12
H–Ala	Glu	Lys	Lys	Asp	Glu	Gly	Pro	Tyr	Arg	Met	Glu

13	14	15	16	17	18	19	20	21	22
His	Phe	Arg	Try	Gly	Ser	Pro	Pro	Lys	Asp–OH

Relationships between chemical structure and biological activity

The amino acid sequences of human, pig and ox β MSH s and of pig α MSH are given in Fig 3 As shown, human β MSH differs from the other two β MSH s in two important

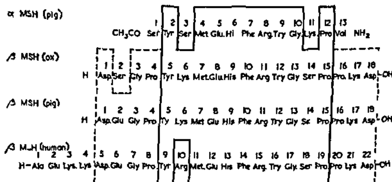


FIG 3 Amino acid sequences of melanocyte stimulating hormones from pig ox and human pituitary glands

respects In the first place, the lysine residue which occurs in position 6 in the pig and ox hormones is replaced by arginine (position 10) in human β MSH and secondly it contains the tetrapeptide Ala Glu Lys Lys in addition to the basic octadecapeptide structure of the other two hormones

Variations of amino acid sequence occurring in structurally

REFERENCES

DU VIGNEAUD V (1954–5) *Harvey Lect* 50 1
HARRIS J I (1959*a*) *Biochem J* 71 451
HARRIS J I (1959*b*) *Nature (Lond)* 184 167
HARRIS J I SANGER F and NAUGHTON M A (1956) *Arch Biochem Biophys* 65 427
INGRAM V M (1957) *Nature (Lond)* 180 326
LI C H (1958) *In* Symp Protein Structure p 302 ed Neuberger A London Methuen
LIGHT A and DU VIGNEAUD V (1958) *Proc Soc exp Biol (N Y)* 98 692

DISCUSSION

Li Dr Harris is to be congratulated on this piece of work Not everyone may realize how difficult it is to get a complete structure from such a small amount of material This is an outstanding example of the use of ultramicro techniques This is the first instance I know of where with only 5 mg one can really determine the complete sequence of 22 amino acids Now I have one question Is there any indication from the work of Dr Dixon and Dr Lee and co workers of the presence of α MSH in the human pituitary? I know you have mentioned that they have not actually found any but is there any indication of it?

Harris As you know Prof Li α MSH is considerably more basic than β MSH and if it had been present in extracts of human pituitary glands I feel sure that it would have been detected However since the glands had been stored in acetone in which α MSH is known to be soluble it is very likely that any α MSH which was originally present was lost before the glands were worked up to obtain β MSH

Li Previously we had difficulty in locating α MSH in both beef and pig posterior pituitary glands Recently we received a new batch of posterior lobe from a local concern and we have now been able to find α MSH in pig beef and horse posterior pituitaries All these three species of α MSH have the structure proposed by Drs Harris and Lerner In this matter of structural similarities among various species what is particularly exciting about the report we have heard here is that the human ACTH has the same structure as the sheep and the ox ACTH

Harris We cannot be sure that the chemical structures are in fact identical All we can say for the moment is that human ACTH has the same amino acid composition as the sheep and ox hormones

Li This problem of replacement of lysine by arginine in the structure is quite interesting to us We have recently synthesized a pentapeptide in the 7 amino acid active core and it possesses MSH activity If we replace the arginine by ornithine there is no loss of

In the case of β MSH's we have seen that a lysine residue in the pig and ox hormones is replaced by the chemically related amino acid arginine, in the human hormone with this one exception all the other structural features which are common to all known MSH's and corticotropins are preserved in human MSH, which suggests strongly that the amino acids which occupy these key positions (i e 5–15 in pig and ox MSH s 9–19 in human MSH) form a structure which is in some way directly concerned with biological activity at least in the *in vivo* environment in which these substances have been designed to function as hormones The structures formed by the amino acids which occur on either side of these key structures appear to be less specific since as illustrated in Fig 3 considerable variations in structure are possible In addition the *interchange* of lysine and serine residues which occurs between positions 3 and 11 in both α MSH and corticotropins suggests that factors other than the linear arrangement of amino acid residues must be taken into consideration when relating structure to biological function in the living cell even in comparatively small peptide molecules which do not seem to possess any ordered three dimensional structure in aqueous solution

Chemical studies on corticotropin and MSH as well as the earlier studies carried out by du Vigneaud and his associates on the posterior pituitary hormones oxytocin and vasopressin (see for example du Vigneaud 1954–5 Light and du Vigneaud 1958) enable us to formulate certain empirical correlations between chemical structure and the biological specificities of these peptide hormones It is established that a particular hormone molecule (e g oxytocin or corticotropin) defined in terms of a unique chemical structure can manifest more than one biological activity and that the different biological activities can be related to specific sequences of amino acid residues It is to be hoped that future studies will add to our knowledge of the mechanisms by which these peptide hormones act, and of the sequence of biological events which they catalyse in the living cell

BLOOD CONCENTRATION OF THYROTROPIC HORMONE IN NORMAL SUBJECTS AND IN PATIENTS WITH THYROID DISEASE

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THE physiological action of thyrotropic hormone (TSH) and the part it plays in the development of thyrotoxicosis and thyroid adenoma are still largely unknown (Taurog Tong and Chaikoff 1957) It is now established that all the phases of thyroid iodine metabolism are inhibited by hypo physectomy iodide concentration capacity rate of formation of radioactive iodine labelled thyroglobulin and secretion of thyroid hormone into the blood stream As far as hormone synthesis is concerned the most striking inhibition bears on the formation of labelled thyroxine and to a lesser extent of diiodotyrosine

The primary site of action of TSH on thyroid iodine meta bolism has been discussed by several investigators (Rawson 1949, Stanley and Astwood 1949 Bogdanove and Halmi 1953 D Angelo 1955 Taurog Tong and Chaikoff 1957) Current opinion seems to be that changes in iodine metabolism in response to alteration in TSH level are too widespread to be explained by a primary effect on any single phase of iodine metabolism It appears likely according to the experimental results of Stanley and Astwood (1949) and Taurog, Tong and Chaikoff (1957) that the primary action of TSH is on some general and fundamental property of the thyroid tissue such as enzyme synthesis and that all the effects on iodine meta bolism are secondary to this Since Greer s postulation (1952 1957) of the existence of two different thyrotropic hormones— a growth ' factor and a metabolic factor—still awaits confirmation by the isolation of the separate fractions from

activity So apparently you can alter certain amino acids in a sequence without loss of activity

Harris What you say is of considerable interest but nevertheless I feel that one should distinguish clearly between naturally occurring polypeptide structures which have been designed to function as hormones at physiological concentrations and in a particular physiological environment and synthetic polypeptide analogues which although active in the frog assay are nevertheless several orders of magnitude less active than the natural hormones α and β MSH

Li The synthetic pentapeptide had lower specific activity than that of ACTH and MSH

Harris Both Boissonnas and Hofmann have achieved the total synthesis of α MSH and Hofmann for example reports that synthetic fragments comparable to your pentapeptide are from 10^3–10^4 times less active than the natural hormone Furthermore even the complete tridecapeptide without its acetyl group is only 1–2 per cent as active as α MSH itself which shows that one has to acetylate the N terminal serine residue in order to obtain a fully active molecule

Sonenberg Is there some precedent for other biologically active peptides being *N* acetyl?

Harris The only other example that I know of is the protein of tobacco mosaic virus which oddly enough also contains *N* acetyl serine as its terminal residue In this connexion however I would mention that Dr J P Waller in our laboratory has succeeded in acetylating corticotropin selectively on the α NH group of its N terminal serine residue The ACTH activity of the resulting *N* acetylcorticotropin was considerably reduced its MSH activity on the other hand was appreciably enhanced showing that the terminal serine residue plays an important role in determining the relative ACTH and MSH activities of the corticotropin molecule

Young I m sure that we would all agree with Prof Li in congratulating Dr Harris on his achievement in determining the chemical structure of a peptide on 5 mg of material The claims of biological research are often incompatible with the irreversible procedures of the chemist and we also ought to congratulate Dr H B F Dixon on releasing his first 5 mg of material without further biological investigation to the claims of Dr Harris and his chemistry

Li Dr Dixon is a chemist rather than a biologist!

Young He is a biochemist!

Here again we have a delightful example of international co operation Dr Harris comes from Cambridge he has been working in Harvard and he presents on behalf of Dr Lee and co workers of Yale a communication at a conference in Argentina!

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the pituitary, it seems wise to assume for the time being that only one hormone exists with the different known properties If TSH is essential for both the growth and the maintenance of thyroid tissue, it must play an important role in the metabolism of thyroid proteins On the other hand, its action on the series of consecutive reactions leading from the trapping of iodide from the blood to the extrusion of thyroid hormone into the blood stream can be considered as a regulating effect on the rate of formation of the special enzymes which are likely to be required at each step in the process

The response of thyroid tissue to exogenous or endogenous TSH is also subject to modification by a variety of factors reviewed by Rawson (1952) Most interesting is the effect of iodide which in excess usually diminishes the thyroid stimulating action of TSH this effect is not invariable, as shown by Halmi, Spirtos and Stuelke (1955) The effect of antithyroid compounds which enhance the thyroid stimulating action of TSH (Halmi and Spirtos 1954), has been attributed to the destruction of an inhibitory substance in impure preparations of TSH or to the conversion of an inactive material to an active form As yet however there is no satisfactory explanation of these findings and it is possible that an explanation might be based on the dependence of thyroid activity on the rate of action of a special enzymic system Furthermore it is likely that still other factors modifying the thyroid stimulating action of TSH are yet to be discovered

This unique thyroid stimulating property of TSH explains the attempt which has been going on for years to correlate hyperthyroidism with the secretory activity of the anterior pituitary Several authors (Means 1943 1944 1949, Rawson Graham and Riddel 1943, Rawson 1956) have postulated the central origin of the disease i e that a neurohumoral mechanism is involved in which stimuli of increased frequency and intensity pass by way of the hypothalamus to stimulate an increased output of TSH by the anterior pituitary gland Thus, the activity of the thyroid cells is augmented by an overproduction and release of thyroid hormone The excess

of circulating thyroid hormone sets up the characteristic secondary changes throughout the body On the other hand, it appears that the suppressive action of the thyroid hormone upon the secretion of TSH by the hypophysis is no longer sufficient to neutralize the strong stimulation received by way of the hypothalamus the net result being a maintained over activity of the thyroid gland with development of a diffuse toxic goitre of the Graves disease type

This theory finds experimental support in the effects on thyroid function of hypothalamic lesions pituitary stalk section and electrical stimulation of the hypothalamus (see Harris 1948 1955) Yet it does not explain the paradox of hyperthyroidism occurring in cases of panhypopituitarism or hypophysectomy which have been recently described To the case reported by Spence (1947) in which Graves disease developed in a 23 year old woman with pituitary infantilism must now be added the cases described by Fajan (1958) and by Werner and Stewart (1958) Two cases of thyroid adenoma were reported by Gurling Baron and Radley Smith (1959) in hypophysectomized women one of whom became severely thyrotoxic As pointed out by the latter investigators on general principles it is not surprising that thyroid adenomata are autonomous compared with diffuse hyperplastic goitres since benign adenomata in other endocrine glands are known to be independent of the respective tropic hormones The metabolism of the thyroid cell in the adenoma might well differ in type from those in the rest of the parenchyma and thyroid hormone might be elaborated in the presence of the necessary enzymes even in the absence of TSH which is required by the normal cell for the same process This could justify consideration of toxic adenoma as an entity distinct from Graves disease as far as pathogenesis is concerned However the fact that severe thyrotoxicosis can develop in the hypophysectomized human subject supports the contention that thyrotoxicosis is not necessarily a pituitary induced disorder but does not preclude that this might be the case in most instances

The discrepancies concerning the aetiology of Graves' disease can thus be readily understood and the conflicting results obtained in the assay of TSH in human subjects by means of different techniques add even more to the general confusion It seems, however, that the problem will be re solved only by means of the exact estimation of the tropic principle in the blood and by a careful analysis of the results obtained for the assay of TSH in normal and pathological cases

The estimation of TSH in body fluids

The euthyroid state

In considering the normal concentration of TSH in man, the first question to be answered is what is normal? The definition of the normal concentration of TSH is usually based on our ignorance of most of the inter related effects of the other hormones on thyroid activity It seems logical to assume that in terms of the physiological function of the hypothalamo pituitary system differences in age and sex can change in unknown proportions the normal basal level of TSH secretion As a result of their theoretical calculations it was postulated by Greer and Shull (1957) that the concentration of TSH in the blood cannot exceed 6 m units/100 ml From this figure it appears as believed by these authors that *TSH is by no* means measurable in normal human blood unless extracted from large quantities Even if it cannot be measured by means of histological techniques based on acinar cell height, the sensitivity of which appears to be too low to permit even a qualitative detection of the hormone in biological fluids more recent techniques are sufficiently sensitive to allow quantitative estimations

It can be definitively stated that the diagnostic value to the clinician and the physiologist of TSH determinations in biological media is entirely dependent on the reliability of the assay methods employed Accordingly, it is appropriate before dealing with clinical problems here to review briefly

the assay methods which seem more likely to solve the problem of TSH estimation in body fluids

The inability to characterize TSH chemically has pre cluded the development of a chemical method of assay and we must still rely upon biological techniques which are often slow tedious and inconvenient

Although Piotti (1957) claimed that TSH could be esti mated in normal human serum after acetone extraction by means of a modification of Tala s (1952) histoquantitative assay technique it appears that among the non isotopic techniques the stasis tadpole method described by D Angelo and co workers (D Angelo Gordon and Charipper 1942 D Angelo *et al* 1951) is the only histological technique sufficiently sensitive and specific to detect the presence of the hormone in serum The acceleration of metamorphosis in tadpoles after TSH administration has been augmented by total starvation prior to a critical developmental stage Starvation arrests metamorphosis and induces thyroid atrophy so that the response to TSH is increased The analysis of variance of the data on which the standard dose response curve is based for the assay gives an index of precision (lambda) of 0 137 and routinely using 7–9 tadpoles in each bioassay group the method is claimed to differentiate success fully between two doses of TSH in a ratio of 1 35 1 0 (D Angelo and Traum 1958)

The *in vivo* isotopic techniques recently developed can be divided into two groups according to the chosen end point either (1) the uptake of the isotope by the stimulated thyroid gland or (2) the release of labelled thyroid hormone Two isotopes have been considered in the uptake experiments Radioactive iodine uptake by the thyroid gland after previous inhibition of the endogenous TSH secretion by iodo casein feeding has been used by Querido Kassenaar and Lameijer (1953) in mice and by Overbeek and co workers (1953) in rats with a precision index of 0 34 and a sensitivity of 10 μg of provisional U S P standard Having achieved inhibition of the endogenous secretion by administration of thyroid powder

to rats, Levey, Cheever and Roberts (1956) developed an assay technique with a sensitivity of 0 625 m units (U S P) and a lambda of 0 24 Both techniques are much less sensitive than that involving tadpoles, and negative results were obtained by Querido, Kassenaar and Lameijer (1955) after the injection of untreated serum However, these workers have devised a technique similar to that employed by Cohn and co workers (1946) for *concentrating the serum and were able to detect* TSH activity in such extracts

The increased turnover of phosphorus which accompanies cell growth was used as an assay index of thyroid over activity in assay techniques employing radioactive phosphorus (Borell and Holmgren 1949 Lamberg, 1952 1953 1955, Greenspan *et al* 1956) The sensitivity of these techniques is of the order of that achieved with the use of radioactive iodine 0 5 m units (Lamberg 1955) and 0 25 m units (Greenspan *et al*, 1956) the latter with an index of precision of 0 34

The release of labelled thyroid hormone after the adminis tration of TSH has proved in recent years to be a good index of thyroid activity It was developed by Adams and Purves (1953, 1955) in the guinea pig, and by Gilliland and Strudwick in the chick (1953) and was further used successfully by Bates and Cornfield (1957) in the chick and by McKenzie *(1958a) in the mouse The general sensitivity of the release* methods is high [0 1 m units (Adams and Purves 1955) and 0 15 m units (Gilliland and Strudwick 1953)], while McKenzie (1958*a*) achieved a sensitivity of 0 025 m units for an injection volume of 0 5 ml with a lambda of 0 24

A further increase in sensitivity has been sought by the introduction of *in vitro* assay techniques Tissue culture experiments have several advantages over *in vivo systems* for the detection of TSH

(i) the added TSH can be confined in a small volume with the target tissue avoiding dilution throughout the body as happens with *in vivo* techniques

(ii) only the isolated target tissue is present, avoiding catabolism of TSH by other tissues and the inter related effect of other endocrine glands
(iii) a certain amount depending on the size of the organ of comparable fragments of the same origin can be prepared

An *in vitro* assay based on the depletion of previously stored labelled thyroid hormone was devised by Bottari and Donovan (1957) and will be fully described elsewhere The sensitivity achieved by the use of this technique is of the order of 0 1 m units/100 ml with an average index of precision of 0 13 The weight gain of incubated bovine thyroid slices under the action of added TSH was used as an index of thyrotropic activity in the assay technique described by Bakke and co workers (1957) Although the sensitivity and the pre cision index of the method are comparable to those of the *in vitro* depletion technique it is not useful for the estimation of TSH in body fluids (Bakke and Lawrence 1959)

The ideal method for assaying TSH has probably not been devised yet From the point of view of sensitivity the *in vitro* techniques appear to be about ten times more sensitive than the *in vivo* methods with a better precision index The *in vitro* depletion technique of Bottari and Donovan (1957) which has proved useful for the estimation of the TSH con tent of the blood both in the human and in animals has the disadvantage of being greatly dependent on the strain of guinea pigs used Work is still in progress to establish fully what are the requisites of the technique in order to avoid any possible difficulty in the interpretation of the results obtained

The statement of Albert (1949) that TSH has not been un equivocally demonstrated in blood must now fortunately be revised Limiting our review of the figures obtained for the TSH content of body fluids in the euthyroid state in man to the quantitative estimations based on the use of reliable assay methods it now seems to be established that the hormone has been detected in blood and in urine at least in a certain number

of cases It is of interest that, despite the variety of the techniques used the data now available in the literature are in close agreement

In the early reports of Querido and Lameijer (1956) and Gilliland and Strudwick (1956) the normal concentration of TSH in blood was considered to be between 0 and 20 m units/100 ml serum Without specification either of the number of cases investigated or of any sex or age differences a mean figure of 40 m units/100 ml was given for normal adult sera by Di George D'Angelo and Paschkis (1957) while the normal figure was again estimated by McKenzie (1958*a*) to be around 20 m units/100 ml A tentative reappraisal of the problem was made by the investigation of a large group of normal human subjects of different age and sex by means of the *in vitro* depletion technique Initial estimations were carried out on a homogenous group of 60 20 year old males Thyrotropic activity was demonstrable in the serum of all of them and the mean concentration observed was 29 m units/100 ml (International Thyrotropin Standard) with outside limits of 8 and 80 m units/100 ml Extension of the male group up to the age of 60 by the addition of 50 new cases did not change significantly the mean concentration previously calculated This was established to be 25 m units/100 ml with outside limits of 7 and 80 m units/100 ml serum (Fig 1)

Sex differences and the eventual inter related effect of oestrogenic activity on the level of circulating TSH was further explored by the study of 64 euthyroid women divided into two groups a group of 36 premenopausal women from whom blood was collected without correlation with the menstrual cycle and a group of 28 postmenopausal women under the age of 60 years The mean value calculated for the first group was 60 m units/100 ml serum with outside limits of 30 and 100 m units/100 ml while the mean concentration calculated for the second group was 23 m units/100 ml with outside limits of 8 and 45 m units/100 ml (Fig 2) Although the number of cases investigated was smaller than in the male group there was a statistically significant difference

between the two female groups The postmenopausal values were comparable to the figures from the male group while the premenopausal mean value was more than twice as high as the male value

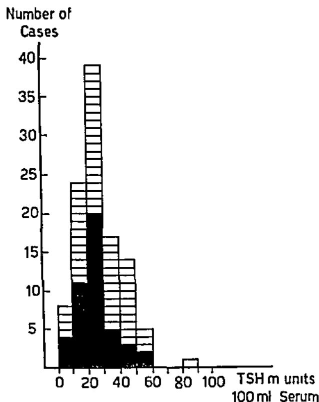


FIG 1 Individual TSH concentration in blood of 110 normal males

□ 0 y r ld male
■ 20–60 year-old m les

As might be expected less clearcut pooled results were obtained from the study of TSH concentration in the blood of 20 individuals of both sexes over the age of 60 years It appears from these few cases that there is a downward trend in this age

group, since sometimes no thyrotropic activity was detectable and most of the results ranged from 5 to 8 m units/100 ml, nevertheless, some higher figures were obtained including a value of 50 m units/100 ml for a 65 year old man

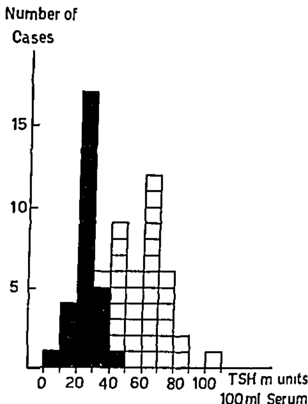


FIG 2 Individual TSH concentration in blood of 64 normal females

☐ premenopausal
■ postmenopausal under the age of 60 years

It is interesting to compare these figures obtained for blood TSH content with the data published by Bakke and Lawrence (1959) for the pituitary TSH content in the human The mean concentration for subjects under the age of 50 years was

210 m units/100 ml while for the older group the mean con centration went down to 82 m units/100 ml The ratio between the pituitary and the serum concentration is compar able for the two age groups although it appears that a dif ference arises later as far as the blood TSH values are con cerned Confirmation of the blood TSH concentration in euthyroid males was obtained by El Kabir (1959) using the *in vitro* assay technique described by Bottari and Donovan (1957)

Some determinations of TSH concentration in urine have been reported in the literature The questionable specificity of the assay method used renders the figures reported by Aron (1951) for untreated specimens rather doubtful The values obtained by Bloch Michel and Henry (1955) for TSH in urine appear however to be in good agreement with the serum values Detectable amounts of TSH were also found in the urine by Savoie (1951) in 10 out of 17 euthyroid cases The five positive responses obtained by Greenspan and co workers (1956) using the method of ^{3} P uptake are more dubious since a recent re evaluation by Greenspan and Lew (1959) of the assay technique has shown that it is not applicable to the estimation of TSH in body fluids

Considering the high toxicity of untreated urine specimens and the possibility that TSH might be metabolized in the kidney (D Angelo 1956 Kassenaar Lameijer and Querido 1956 Kassenaar Kerkhofs and Querido 1959) estimations of TSH *in blood appear to be a more reliable index of thyro* tropic activity than estimations in urine

TSH concentration in hypothyroidism

It has been considered for years that myxoedema is accompanied by an increase in TSH concentration in the blood sufficient to permit its estimation even by the techniques based on acinar cell height Positive qualitative findings were reported by Hertz and Oastler (1936) Emerson and Cutting (1938) and Collard and co workers (1940) It was recently *shown however (Gilliland and Strudwick 1956*

group, since sometimes no thyrotropic activity was detectable and most of the results ranged from 5 to 8 m units/100 ml nevertheless, some higher figures were obtained, including a value of 50 m units/100 ml for a 65 year old man

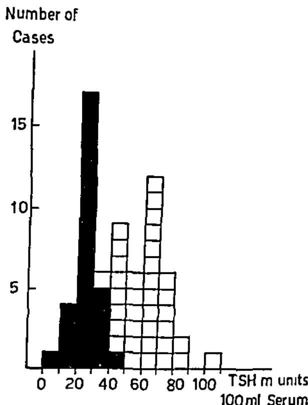


FIG 2 Individual TSH concentration in blood of 64 normal females

□ premenopausal.
■ postmenopausal under the age of 60 y rs

It is interesting to compare these figures obtained for blood TSH content with the data published by Bakke and Lawrence (1959) for the pituitary TSH content in the human The mean concentration for subjects under the age of 50 years was

thyroid patients Increased amounts were found in 17 out of the 41 cases collected in the literature while normal or decreased amounts were found in the others An increased out

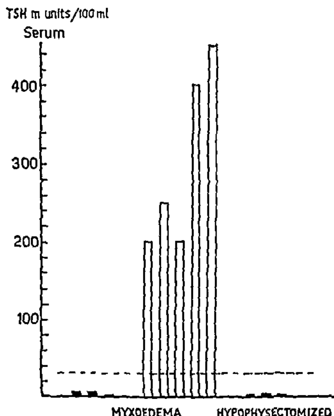


FIG 3 Individual results observed in cases of spontaneous myxoedema and after hypophysectomy

– normal verage concentration of TSH in blood

put of TSH was observed in 11 out of 26 urinary estimations Another interesting feature was the report by Adams (1958) later confirmed by McKenzie (1958*b*) of the existence in the blood of some thyrotoxic patients of an abnormal thyrotropic

D'Angelo *et al*, 1951 Di George *et al*, 1957) that this is not always the case and that absence of thyrotropic activity can be demonstrated in severe cases of hypothyroidism The finding that TSH activity is restored in those cases by thyroid therapy is an interesting one (Starr *et al*, 1939, Salter 1940) Quantitative estimations of the TSH content of myxoedematous blood have now been performed by means of four different assay techniques Although the ^{131}I discharge from the thyroid of the one day old chick used by Gilliland and Strudwick (1953) does not always measure the TSH content of normal blood elevated TSH titres were found by this method in 8 out of 10 patients with spontaneous myxoedema The results reported by D'Angelo and co workers (1951) and Di George and co workers (1957) also demonstrated that the TSH content of myxoedematous blood varies widely from patient to patient being either undetectable normal or excessively high Values of up to 400 m units/100 ml were obtained in untreated patients while undetectable amounts increased up to 200 m units/100 ml after thyroid therapy in other patients Slightly supranormal figures were observed by McKenzie (1958*a*) in two out of four cases

The seven cases of spontaneous myxoedema investigated in Brussels by means of the *in vitro* depletion technique are consistent with the previous reports Four of them had an abnormally high serum TSH level 200 250 450 and 400 m units/100 ml while the three other cases had low values undetectable in one and only 4 m units/100 ml in the other two (Fig 3) Since it was not possible to examine the response of the thyroid of these patients to exogenous TSH one can of course not exclude the possibility of a primary deficiency of TSH in these patients A serum concentration of 200 m units/100 ml was found by El Kabir (1959) in a case of spontaneous myxoedema

TSH concentration in hyperthyroidism

Adams and Purves (1957) have recently reviewed the results of TSH estimations performed in the blood of hyper

thyroid patients Increased amounts were found in 17 out of the 41 cases collected in the literature while normal or decreased amounts were found in the others An increased out

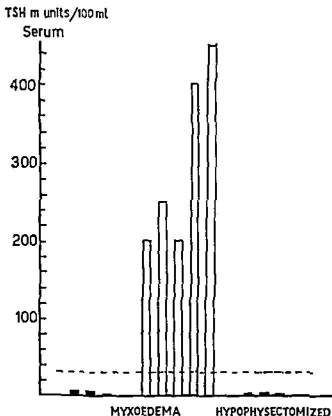


FIG 3 Individual results observed in cases of spontaneous myxoedema and after hypophysectomy

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put of TSH was observed in 11 out of 26 urinary estimations Another interesting feature was the report by Adams (1958) later confirmed by McKenzie (1958*b*) of the existence in the blood of some thyrotoxic patients of an abnormal thyrotropic

hormone characterized by its delayed action when injected into the test animal

The concentration of TSH in the blood was estimated by means of the *in vitro* depletion technique in a great number of

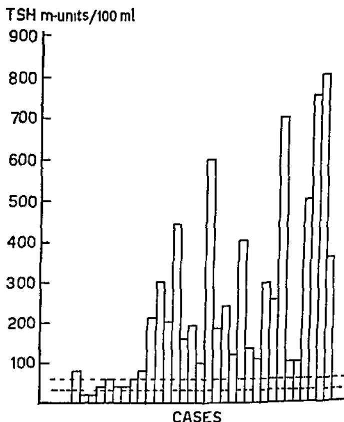


FIG 4 Individual TSH concentrations in blood of thyrotoxic patients

- - - normal limits of variation

patients with a subjective symptomatology of thyrotoxicosis The diagnosis was separately assessed by classical means physical examination basic metabolic rate determinations ^{131}I uptake and sometimes the calculation of the conversion

ratio No abnormal TSH value was observed in the cases diagnosed as euthyroid by classical investigations The figures observed for the 45 truly thyrotoxic cases may be

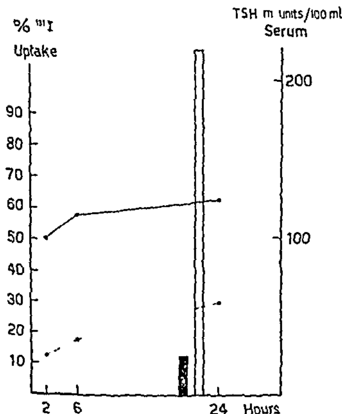


FIG . Comparison of ^{131}I uptake and TSH concentration in blood of thyrotoxic patient

■ --- average figures observed in euthyroidism
□ —— values observed in thyrotoxic patient

divided into three groups A first group of 9 patients had TSH blood concentrations in the normal euthyroid range 23 patients were found to have an increased concentration ranging from 100 to 800 m units/100 ml serum, the third group of

18 patients was characterized by the tremendously high concentration observed, all the values obtained being higher than 1 *unit/100 ml* *Individual* results are illustrated in *Figs* 4 and 5 Two of the three thyrotoxic cases observed by El Kabir (1959) had concentrations of about 200 m units/ml, and the third had a normal concentration

The data so far obtained do not permit us to establish a correlation between the severity of the thyrotoxic state and the concentration of TSH in the blood Even if we consider these data as provisional, it is striking that in none of the cases examined was the TSH serum level diminished, which confirms the observations of D Angelo and co workers (1951)

Estimations of TSH in the blood were also carried out in a few patients before and after the diagnostic administration of triiodothyronine The marked drop observed in the blood TSH concentration for those cases was accompanied by a parallel decrease in the thyroid ^{131}I uptake

DISCUSSION

The supposition that the thyroid and the pituitary are reciprocally related has led many workers to attempt the detection and definition of TSH in the body fluids of normal subjects and of thyroid patients The results however have been largely inconclusive and confusing and have long obscured the study of the physiological and pathological role of the hypophysis in thyroid disease It is now obvious that the discrepancies regarding the presence or absence of TSH in blood or urine were due to the crude methods used to detect the low titres of hormone present in body fluids The techniques recently devised in some laboratories have now seemingly achieved the degree of sensitivity precision and specificity required for a complete reinvestigation of the problem The first aim of the present study was a tentative definition of the range of concentration of TSH in the untreated serum of normal individuals in different age and sex groups In confirmation of previous observations it is now established

that endogenous TSH can be detected in blood despite its low concentration From the results obtained in a group of 110 healthy males ranging from 20 to 60 years of age this normal concentration was estimated to be about 25 m units/100 ml serum The interesting finding that the concentration of TSH in the blood of premenopausal women is more than twice as high as that in males should be confirmed on a larger population and perhaps correlated with the menstrual cycle

The existence of a fine balance between the pituitary and the thyroid has been repeatedly suggested by several investigators This concept was supported by the accumulation of much indirect evidence Studies based on the assay of TSH in blood using reliable techniques do not provide the results one would expect if a reciprocal regulatory system were operating between the two glands The results obtained by means of the *in vitro* depletion technique in myxoedematous patients confirm the earlier findings of D Angelo and of Gilliland and Strudwick indicating that a low serum titre of thyroid hormone is not always paralleled by a high TSH serum concentration This finding is not surprising since Eartly and Leblond (1954) have shown that thyroidectomy in the rat was followed by a decreased secretion of most of the tropic hormones (growth hormone adrenocorticotropic hormone and gonadotropins) The present author has observed (unpublished data) that in the rabbit thyroidectomy is followed after a transitory phase of pituitary TSH overproduction by a gradual fall of the TSH content of the blood The reappearance of TSH in the circulation after thyroid therapy reported by Di George and co workers (1957) is attributed by them to a general bodily improvement with heightened metabolic activity and renewed ability to synthesize and release TSH

On the other hand in hyperthyroid patients known to have a characteristically high thyroid hormone level TSH values in blood were found to be either normal or elevated The question arises whether the high amounts found in some patients are not due to some artifact possibly the presence of some toxic substance noxious to the incubated thyroid tissue

13 patients was characterized by the tremendously high concentration observed all the values obtained being higher than 1 unit/100 ml Individual results are illustrated in Figs 4 and 5 Two of the three thyrotoxic cases observed by El Kabir (1959) had concentrations of about 200 m units/ml, and the third had a normal concentration

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BLOCH MICHEL, H and HENRY R (1955) *Ann Endocr (Paris)* **16** 268

BOGDANOVE E M and HALMI N S (1953) *Endocrinology* **53** 274

BORELL, U and HOLMGREN H (1949) *Acta endocr (Kbh)* **3** 331

BOTTARI P M and DONOVAN B T (1957) *J Physiol (Lond)* **140** 36P

BOTTARI P M DONOVAN B T and EL KABIR D J (1959) Unpublished data

COHN E J STRONG L E HUGHES W L Jr MULFORD D J Ashworth J N MELIN M and TAYLOR H L (1946) *J Amer chem Soc* **68** 459

COLLARD H B MILLS F H RUNDLE F F and SHARPEY SHAFER E P (1940) *Clin Sci* **4** 323

D ANGELO S A (1955) *Brookhaven Symp Biol* **7** 9

D ANGELO S A (1956) *J clin Endocr* **16** 976

D ANGELO S A GORDON A S and CHARIPPER H A (1942) *Endocrinology* **31** 217

D ANGELO S A PASCHKIS K E, GORDON A S and CANTAROW A (1951) *J clin Endocr* **11** 1237

D ANGELO S A and TRAUM R E (1958) *Ann N Y Acad Sci* **72** 239

DI GEORGE A M D ANGELO S A and PASCHKIS K E (1957) *J clin Endocr* **17** 842

EARTLY H and LEBLOND C P (1954) *Endocrinology* **54** 249

EL KABIR D J (1959) Personal communication

EMERSON K Jr and CUTTING W C (1938) *Endocrinology* **23** 439

FAJAN S S (1958) *J clin Endocr* **18** 271

GILLILAND I C and STRUDWICK J I (1953) *Clin Sci* **12** 265

GILLILAND I C and STRUDWICK J I (1956) *Brit med J* **1** 378

GREENSPAN F S KRISS J P MOSES L E and LEW W (1956) *Endocrinology* **58** 767

GREENSPAN F S and LEW W (1959) *Endocrinology* **64** 160

GREER M A (1952) *J clin Endocr* **12** 1259

GREER M A (1957) *Ciba Found Coll Endocr* **10** 34 London Churchill

GREER M A and SHULL, H F (1957) *J clin Endocr* **17** 1030

GURLING K J BARON D N and RADLEY SMITH E J (1959) *J clin Endocr* **19** 717

HALMI N S and SPIRTOS B N (1954) *Endocrinology* **55** 613

HALMI N S SPIRTOS B N and STUELKE R G (1955) *Endocrinology* **57** 502

HARRIS G W (1948) *Physiol Rev* **28** 139

HARRIS G W (1955) Neural Control of the Pituitary Gland London Edward Arnold

HERTZ S and OASTLER E (1936) *Endocrinology* **20** 520

KASSENAAR A A H KERKHOFS H and QUERIDO A (1959) *Acta endocr (Kbh)* **31** 171

KASSENAAR A A H LAMEIJER L D F and QUERIDO A (1956) *Acta endocr (Kbh)* **24** 35

This last point deserves further investigation and might well be correlated in some way with the abnormal hormone described by Adams (1958) However it is apparent from these results that if thyrotoxicosis is not necessarily of pituitary origin the repeated finding that TSH exists either in normal or in elevated concentrations in the blood of thyrotoxic patients strongly suggests that it must play an important rôle in the onset and maintenance of Graves disease in most instances

It is of interest that according to Bakke and Lawrence (1959) the concentration of TSH in the pituitary found in the only thyrotoxic case examined was as high as 1 100 m units/100 ml for a mean value of 114 m units/100 ml

Summary

Serum levels of thyrotropic hormone (TSH) in euthyroid adults of different age and sex and of hypothyroid and hyperthyroid patients are reported The normal concentration of TSH in the blood of men and of postmenopausal women under the age of 60 years was found to be 25 m units/100 ml, while a concentration of 60 m units/100 ml was obtained in the blood of premenopausal women Hypothyroidism was characterized either by abnormally low or by abnormally high serum concentrations Hyperthyroidism on the other hand was accompanied by normal or by abnormally elevated concentrations of TSH in the blood

REFERENCES

ADAMS D D (1958) *J clin Endocr* 18 699
ADAMS D D and PURVES H D (1953) *Proc Univ Otago med Sch* 31 38
ADAMS D D and PURVES H D (1955) *Endocrinology* 57 17
ADAMS D D and PURVES H D (1957) *Metabolism* 6 26
ALBERT A (1949) *Ann N Y Acad Sci* 50 466
ARON M (1951) *Ann Endocr (Paris)* 12 994
BAKKE J L HEIDEMAN M L Jr LAWRENCE N L and WIBERG C (1957) *Endocrinology* 61 352
BAKKE J L and LAWRENCE N L (1959) *J clin Endocr* 19 28
BATES R W and CORNFIELD J (1957) *Endocrinology* 60 225

Another point of considerable interest concerns the primary and secondary myxoedema results which you have shown Drs Gilliland and Strudwick (1956 *loc cit*) working in our department showed that in myxoedematous subjects a small dose of thyroxine would restore the occasional subnormal TSH levels to the supranormal levels you might expect in the presence of myxoedema This was also shown for the urinary gonadotropin Perhaps myxoedema of the pituitary is a reasonable explanation for this finding

I was very interested in the results on thyrotoxicosis and to see such a large and extensive incidence of mostly high values We like many others have tried to divide thyrotoxicosis into groups and I realize that it is very difficult Unfortunately the clinicians have called primary what is really secondary and secondary what is really primary but the question of the presence or absence of exophthalmos has always been very closely connected with the problem and I would be most interested to know whether the values which were low in your series were cases in which the exophthalmos was not recognizable Such a grouping is of course not 100 per cent correct for we have all been very familiar with cases where the exophthalmos is obvious a year later though not when the patient was first seen Equally so the presence or absence of nodules in the thyroid is not very relevant because a nodule in the goitre does not prevent one from getting diffuse hyperthyroidism or what I like to call Graves disease Of course in this connexion we would be most interested to see whether you have been able to demonstrate the hollowness of the Werner logic in saying that the failure to suppress the ^{131}I uptake by the thyroid with T3 or any other thyroid hormone means that the disease is in the thyroid (Werner S C (1954) *J clin Endocr* 14 1260) This finding surely means that some linking in the pituitary thyroid control mechanism is not working normally

Bottari Unfortunately I cannot answer your first question Tests for parallelism between hyperthyroid sera and the TSH standard proved to be conclusive

Russell Fraser Is it not liable to change very much in hyperthyroid patients?

Bottari The assays for parallelism were performed only on sera collected in hyperthyroid or thyroidectomized patients since the content of normal serum is too low to allow of serial dilution

Russell Fraser The pregnant woman would be the one to check

Bottari I agree on the point that pregnancy and cord samples are the cases where it should be looked for As far as the injection of thyroxine in cases of myxoedema with low TSH is concerned I have had no occasion to try it since I am given the blood samples without

LAMBERG B A (1952) Nord endokrinologkongr Helsingfors p 18
LAMBERG B A (1953) *Acta med scand* suppl 279
LAMBERG B A (1955) *Acta endocr (Kbh)* 18 405
LEVEY H A CHEEVER E and ROBERTS S (1956) *Endocrinology* 58 420
MCKENZIE J M (1958*a*) *Endocrinology* 62 865
MCKENZIE J M (1958*b*) *Endocrinology* 63 372
MEANS J H (1943) *Ann intern Med* 19 567
MEANS J H (1944) *Amer J med Sci* 207 1
MEANS J H (1949) *Lancet* 2 543
OVERBEEK G A FOKKENS J QUERIDO A DE VISSER J and CANNINGA P (1953) *Acta endocr (Kbh)* 14 285
PIOTTI L E (1957) *Arch Sci med* 104 471
QUERIDO A KASSENAAR A A H and LAMEIJER L D F (1953) *Acta endocr (Kbh)* 12 335
QUERIDO A KASSENAAR A A H and LAMEIJER L D F (1955) *Ned T Geneesk* 98, 44
QUERIDO A and LAMEIJER L D F (1956) *Proc roy Soc Med* 49 209
RAWSON R W (1949) *Ann N Y Acad Sci* 50 491
RAWSON R W (1952) *Ciba Found Coll Endocr* 4 294
RAWSON R W (1956) *In* The Thyroid a Fundamental and Clinical Text book ed Werner S C London Cassell
RAWSON R W GRAHAM R M and RIDDEL C B (1943) *Ann intern Med* 19 405
SALTER W T (1940) The Endocrine Function of Iodine Harvard University Press
SAVOIE J L (1951) *Ann endocr (Paris)* 13 81
SPENCE A W (1947) *Proc roy Soc Med* 40 151
STANLEY M M and ASTWOOD E B (1949) *Endocrinology* 44 49
STARR P RAWSON R W SMALEY R E DOTY E and PATTON H (1939) *West J Surg* 47 65
TALA P (1952) *Acta endocr (Kbh)* suppl 9
TAUROG A TONG W and CHAIKOFF I L (1957) *Ciba Found Coll Endocr* 10 59
WERNER S C and STEWART W B (1958) *J clin Endocr* 18 226

DISCUSSION

Russell Fraser Dr Bottari is to be congratulated on his very interesting paper It is not often possible to see such a large number of assay results for TSH To come to details Dr Bottari how sure are you that the thyroxine binding protein which you add with the serum has no influence on the assays? One would imagine that this might influence the assay end point The question arises particularly in the case of results which show such a high value for TSH in pregnancy when we know there is a high thyroxine binding protein

start a colony with suitable animals in order to control diet tempera ture and so on

Beck Dr Bottari would you also comment on the possible seasonal variations in guinea pigs? This is seen in other animal species in which you attempt to do TSH assays

Bottari I have got seasonal variations characterized by a wide scatter in the early spring while in London the same phenomenon was observed during winter

Read Dr Bottari have you assayed the serum of patients receiving antithyroid growth hormone?

Bottari Since I was looking for pure results this point was neglected Some patients however have been examined after ^{131}I therapy From the few cases observed it seems that one might conclude that when treatment is followed by a lack of subjective relief TSH remains high although thyroid function appears to be stabilized at a euthyroid level as far as the tracer is concerned

Anderson Prof Russell Fraser I was struck by the exophthalmos of the patient you showed us did you find on more careful examination that the patient had hyperthyroidism?

Russell Fraser No possibly excess of output of TSH but not hyperthyroidism

Anderson Do you feel that exophthalmos is due to the TSH? Drs Bates and Condliffe in our laboratory have two sources of TSH one is beef pituitary and the other is a mouse pituitary tumour which elaborates TSH they have had these preparations assayed for exophthalmic factor by Dr Albert at the Mayo Clinic who uses the fundulus (Bates R W Albert A and Condliffe P G (1959) *Endocrinology* 65 860) The mouse pituitary tumour TSH does not have the exophthalmic factor but it is of course a very potent TSH preparation and the bovine preparation has both TSH and the exophthalmic factor Here is rather clearcut evidence of two factors —the exophthalmic factor and TSH

Bottari Dr El Kabir in London started to look for TSH in exophthalmos He has a case of exophthalmos without thyrotoxicosis and he has found a very low amount of TSH

Russell Fraser Drs Gilliland and Strudwick when they were assaying for these same compounds in the plasma by a less sensitive method divided their patients into three groups one of which was thyrotoxicosis without evidence of exophthalmos another thyrotoxicosis with gross exophthalmos and the third gross exophthalmos without obvious hyperthyroidism We came to the conclusion as regards the latter that there are perhaps two different diseases which masquerade under the one name of exophthalmos In our clinic it is the practice to try suppression tests on all these patients with

having any access to the patients, but I think it is important to look for this in the future

As expected by Prof Russell Fraser all the cases with normal TSH blood levels showed no obvious signs of exophthalmos My experience of large exophthalmos in hyperthyroidism is limited to one single case but since that one had been treated for years I would not like to comment on it

Now as far as Dr Werner s theory is concerned I have in two *instances measured* TSH *in the blood* of *patients where the T3 test* was used and in both of them TSH remained high after the administration of T3

de la Balze Did you try tri iodothyronine in normal human beings to see what happened with TSH?

Bottari One of my slides showed a normal—although his ^{131}I uptake was high This was one of these cases where Werner makes a difference between thyrotoxicosis and euthyroidism

de la Balze How do you explain the low TSH in postmenopausal women?

Bottari I have no real explanation It has been shown that if oestrogens are given to animals inhibition of thyroid function results so I wonder if what one gets is not just a higher level of TSH to maintain the same thyroid function level when there is inhibition by oestrogens It would be interesting to explore this during the cycle but I did not find any volunteers to correlate the blood collected at different periods of the cycle This is what should be done in order to know exactly the correlation between TSH and ovarian function

Loraine The method of McKenzie (1958*a loc cit*) which you mentioned seems to be open to the criticism that sometimes there is significant deviation from parallelism when the standard TSH is assayed against extracts prepared from human serum I wondered if with your method significant slope differences were likely to occur

Would you comment on the necessity for rigid standardization of the guinea pigs which you use in your assay procedure? Is it not the case that only certain strains of guinea pig are suitable and that for some time before use the animals must be kept under very constant environmental conditions? I should add that Miss J R Brown working in our laboratory has been using your method and has obtained results similar to those you have reported However she found that the success of the assay depended to a large extent on the strain of animal used

Bottari The question of the strain of guinea pigs to use is the black spot of the *in vitro* technique The only way to cope with it is to

Normal and reasonable amounts of thyroxine or triiodothyronine will suppress 100 per cent of normals but not in these exophthalmos cases We do get some suppression which we like to call a partial suppression

Sonenberg I agree I think Graves disease is a disease as Prof Russell Fraser said and we too have done the same thing If you give large enough doses these patients can be suppressed and that they do not respond to normal doses may be because either (i) their homeostatic mechanisms are not operative wherever they may be or (ii) it may well be that in those patients who get the same dose as a euthyroid individual the thyrotoxic individual disposes of his thyroid hormone much more rapidly so for all practical purposes he does not have the same level of hormone in his blood to suppress the pituitary But whatever the mechanism there are alternative interpretations of the results other than Werner s

Bottari I completely agree with this

Sonenberg I would add with regard to McKenzie s so called TSH which I think Dr Bottari took a little refuge in—I think he is unduly cautious about this—there is no chemical evidence of any differences in TSH Of course it is too early to tell since no one has ever had pure TSH There are alternative explanations which might explain McKenzie s data which would not include any real TSH namely differences in absorption or binding to other proteins in the serum that you inject and other explanations so you could well have real TSH there whose apparent activity is increased

Raben I think McKenzie s data is harder to explain away than that The factor in the blood of some hyperthyroid patients differed from TSH in several ways It survived in the blood stream of the mouse much longer than injected TSH did it ran differently on electrophoresis and had a distinctly different time curve in discharging radioactive iodine This evidence makes a rather strong case for a qualitatively different substance Indeed I wonder whether Dr Bottari has not chosen a risky time to attribute hyperthyroidism to the pituitary Dr Pearson and others have seen cases of hyperthyroidism without a pituitary and this factor has not yet been found in either animal or human pituitary glands

Bottari The point I want to make is not that hyperthyroidism is always of pituitary origin but that if TSH is found to be increased in a large group of thyrotoxic patients the fact has by no means some significance Indeed I do not see any explanation for an increased TSH blood level in patients known to have a high thyroid hormone blood level One would expect a low TSH since even a normal TSH content should imply that the feed back mechanism is no longer functioning at a normal rate

apparently euthyroid exophthalmos and in our experience nearly all if not all of them do not suppress—they behave like patients with a thyrotoxic gland in that respect even if they have no increased output of thyroid But there are apparently other cases with no hint whatsoever of any involvement of the thyroid with the grossest of exophthalmos of which we have seen a few who respond dramatically to X rays applied to the orbit and I feel that this may not always be related to Graves disease In answer to your first question I was trying to hint in presenting the previous picture that exophthalmos did not only depend on TSH It may be that there are other pituitary hormones which are rather important and that the so called exophthalmos producing factor may be a combination and not a single hormone I would like you to tell me what the other one is!

Sonenberg I have two questions about technique First I wonder if there is a standard curve which compares giving TSH alone and then TSH in serum Secondly it has been reported that when iodides are added to a medium of the kind that you describe they interfere with TSH action This may be just a dose effect—in other words the dose previously used I suspect were much larger than those that you use but I wonder if this point has been checked?

Bottari As far as my first slide was concerned this was standard TSH not TSH in serum Of course you always have serum in the incubation medium We were rather concerned about the sodium iodide with the dose level we were giving there was no effect but at higher dose levels there was an effect

A B Houssay We have done Werner s test in the three groups mentioned by Prof Russell Fraser i e exophthalmic patients with hyperthyroidism and malignant exophthalmos with or without hyperthyroidism and measured the response by the depression of ^{131}I uptake conversion rate and protein bound ^{131}I There was no depression in any of these three tests in hyperthyroid patients but we found generally some depression in exophthalmic patients who have never been hyperthyroid

Russell Fraser This is the difficulty about what you call a positive response to a suppression test My own interpretation of a suppression test is as follows if you give a normal amount of hormone you would expect the function of the gland to go into subnormal levels It does not matter whether that involves a suppression to 50 or 30 per cent of the starting value—the important thing is the absolute value which you achieve at the end When we do these suppression tests we give a slightly larger dose than Dr Werner does We do find some suppression in thyrotoxicosis with a large dose of thyroxine we have suppressed the uptake of some thyrotoxic glands

firmed this observation or if you have any data pertaining to the urinary excretion of TSH in normal and pathological conditions

Bottari I have not tried TSH in urine because I do not believe very much in TSH in urine

Russell Fraser Dr Bottari do you believe in the existence of a TSH inhibitor which has been hinted at by various people, and if this were so would it not be a good way of explaining the abnormal TSH in hyperthyroidism?

Bottari I have no idea on that point

Raben I wonder Dr Bottari if there is this qualitatively different fact and whether those methods which do not depend on a time curve would distinguish it from TSH?

Bottari I don t know but I think that the fact that McKenzie is finding it as a delayed action in animals might also be an explanation

Pearson The observations that we have made of persistent thyroid function following hypophysectomy do not I believe rule out the possibility that thyrotoxicosis may in some cases, be due to excessive TSH production We have observed 10 patients who have remained euthyroid for periods of 6 months to more than 2 years following a complete or nearly complete hypophysectomy Administration of triiodothyronine failed to induce suppression of thyroidal ^{131}I uptake or in serum protein bound iodine levels The majority of these patients had functioning adenomata of the thyroid gland The non adenomatous thyroid tissue appeared to be quiescent and could be stimulated to increased function by administration of TSH Two patients had a previous history of thyrotoxicosis but were euthyroid at the time of hypophysectomy It still seems possible however that thyroid function might be suppressed by hypophysectomy in some patients with hyperthyroidism

Russell Fraser One small point with regard to exophthalmos there is a prevailing tendency to regard it as the same thing at all levels of hyperthyroidism We once had a patient with widespread thyroid carcinoma who had the lid retraction pattern of exophthalmos which some people would call thyrotoxic exophthalmos and when he had his pituitary removed he still had this exophthalmos As long as he was given antithyroid drugs he was not thyrotoxic In one or two other patients the pituitary has been attacked for severe exophthalmos—we have had a little experience of that too—and removal of the pituitary certainly seems to have some influence on it The very few isolated cases of active thyroid are best explained as Dr Pearson suggests by nodules which can be independent and they do not bear on the cause of the common cases of thyrotoxicosis which we might best call Graves disease

Raben How long does it take for the TSH and gonadotropins to reappear in myxoedematous patients given thyroid?

Russell Fraser I have not enough information to answer that because our usual practice was to give 14 days of thyroxine at the dose level of 0 1 mg /day after which the second assays were done by then they had returned to the hypernormal levels in all instances in which we tried it—about three I think

Loraine Some years ago a claim was made by workers in Leiden Holland that in a patient with thyrotoxicosis large quantities of TSH were excreted in urine I wonder Dr Bottari if you have con

tion and it is gratifying to have it so conspicuously illustrated by our colloquium

Prof Young s fourth stage that of the synthesis of a hormone and the study of its variants is you may feel barely beginning perhaps not yet really under way but it must come and I wish particularly to emphasize the plea of Dr Gemzell that the structure of the human FSH be worked out and some method be found as soon as the bio chemists can possibly do it either to synthesize this product from the amino acids or to produce an imitation of it This as everyone realizes is one of the points at which clinical results can be obtained in a condition which causes a great deal of heartbreak and distress namely sterility and in which even the partial successes that have already been made are of very great value When I think of my col leagues at the Rockefeller Institute doing marvels with serial ad sorption and with countercurrent separation of hormones I feel that I want to rush right back and tell them what a field they have in which to put their apparatus to work here in the human pituitary hormones

I have not heard in these three days a single paper which seemed to me to represent a dead end in research Nobody has completed his own immediate task and all I can say as a very grateful participant is that I wish the greatest success to all these workers along the lines which they know best how to pick out for themselves

Young We are very grateful to you Dr Corner for your contri bution I still look back with great interest to your paper on the hormonal control of lactation in 1930 (Corner G W (1930) *Amer J Physiol* 95 43)

de Ulhoa Cintra As a clinician I have been very much impressed with all that has gone on and I hope that better methods will be developed for clinical investigation and for physiological and func tional pathology My hopes are that in the next experiments with pituitary hormones something can be done to find methods for determination and one hope is in immunological processes because in all proteins probably not only are there good antigens but also they may have good specificity as far as being able to improve our methods of finding hormones is concerned

Young The importance of immunology in these developments is one that has been well emphasized at this meeting and I am sure that we shall see this subject rapidly grow

de la Balze I would like that in five or ten years through the help advice and friendship of people from different parts of the world we may be able to do work similar to what they are doing now

A B Houssay I would like someone to discuss the relationship of gonadotropins to the adrenals As I suggested previously in the

GENERAL DISCUSSION

Young As we begin this last general discussion I propose to give an opportunity to members of the colloquium who have not presented papers to express their views on any general question relevant to the colloquium and to future research I shall call on them in turn

Corner As all here know I am not a recent worker in this field To tell you how far back in the past I belong I would say that I think I was the first person in the Americas to perceive the lactogenic effect of the pituitary gland but fortunately before publishing my observations found that I had been anticipated by two workers in Strasbourg who were the actual discoverers My only other role in the pituitary field was an unsuccessful attempt fifteen years ago to produce ovulation in monkeys with the pituitary hormones then available You can readily understand then that the opportunity to listen to this colloquium has given me an overwhelming sense of the progress made by these research workers in the last few years

Prof Young stated in his opening address that there are five stages in the ordinary course of an investigation of the endocrine glands and I have heard papers presented here which touch upon every one of the five The first one from our colleague in London which I was happy to see was in the anatomical field goes back even before stage one for Prof Russell Fraser has shown us that in the study of human pituitary endocrinology the very first step surgical extirpation of the gland still requires careful investigation

The second stage that of crude extracts seems now to have been largely left behind although the other part of Prof Young s second stage attempts to relieve the results of extirpation is of course in active progress

The third stage that of chemical isolation is well under way as exemplified by many of the papers among the most notable being that of Dr Harris As a guest participant here I should like to express my admiration for the generosity of the workers in this field in supplying each other with these precious extracts This does not prevail I regret to say in every branch of scientific research Obviously it pays to be generous but it must hurt at times to give up even a few milligrams of something that has taken so long to work up and which the investigator himself might equally well have used I congratulate you on this attitude which obviously prevails over the whole world among workers on human pituitary hormones Such international co operation is much in the spirit of the Ciba Founda

Answers to these questions require observations on changes occurring in the absence of the pituitary From many studies made in hypophysectomized animals we know some of the alterations in carbohydrate and fat metabolism which can be attributed to absence of the metabolic factors in some species but we have almost no comparable information from human subjects In respect to the normal role of growth hormone in protein metabolism in the adult we know even less Recently we have obtained some evidence which we think means that growth hormone does assist in the maintenance of body tissue in the adult animal The young adult rat maintained on a normal diet loses large quantities of nitrogen in the first few days after hypophysectomy This loss can be prevented by treatment with small amounts of growth hormone but not as yet with anything else It would be very difficult to make similar observations in the human subject I know but I would be interested to hear of any observations made in patients hypophysectomized surgically or radiologically or in hypopituitary subjects on changes either in protein metabolism or in carbohydrate or fat metabolism Of course to interpret the findings in hypophysectomized animals or patients observations on effects of purified hormones remain essential but it is possible that the hypophysectomized subject has been somewhat neglected as a source of information in this area

Malinow I agree with Dr Corner that it is very difficult to offer suggestions to such a fine assembly of able endocrinologists who have contributed so much to the advancement of their disciplines Nevertheless as a cardiologist I would like to draw your attention to some of the many problems which might still greatly benefit from co-operative studies

Atherosclerosis for instance is apparently related in the human to functioning gonads since coronary arterial disease is by far more common in men than in women and this difference tends to disappear after the menopause (Malinow M R (1956) *Cienc e Invest* 12 395) Experimental atherosclerosis too can be prevented or made to disappear by oestrogen therapy (Malinow M R (1960) *Acta physiol lat amer* in press) and this may be mediated through blood chemical changes as well as through direct effects of the hormones on the vessels (Malinow M R (1960) *Circulat Res* in press) Furthermore the endocrines may also control blood clotting factors at least in the rat (Gilman T and Naidoo S S (1958) *Endocrinology* 62 92 Torres H N Malinow M R and Mirochnik L (1958) Unpublished data) and this is another mechanism which could *perhaps influence arterial occlusions Endocrine control of* blood lipids can also be probably related to atherosclerosis

Another subject that needs clarification is that of the endocrine

discussion I am interested to know of your experience of the action of gonadotropins upon human adrenal glands I am very happy to take advantage of the generous offer of Prof Wilhelmi in our hypophysectomized rats with adrenal tumours the oestrus disappeared after hypophysectomy and came back after gonadotropins were administered and I would like to receive from Prof Wilhelmi some FSH and LH in order to study the separate influence of both factors

Rodriguez We are very grateful for the great help that some of you have given us in providing us with some of the more or less pure fractions from the pituitary gland

My field of research is related mostly to carbohydrate metabolism and we have seen that some of the hormones from the pituitary gland have diabetogenic action and some have not Working with forced fed alloxan diabetic rats with partially pancreatectomized dogs cats pigeons and turtles, and with normals we have seen that growth hormone is the most potent diabetogenic agent from the pituitary gland The second is prolactin the third is ACTH and FSH gonadotropins and the so called hormones from the posterior pituitary gland have no action in our tests In studying the action of growth hormone we have done a few experiments on the diabetogenic and the growth promoting action and so far we have not seen hormones with diabetogenic action without growth promoting action or *vice versa* and I would like to hear some comments on this

Pearson It is clear from listening to the papers that the need for human pituitary hormones for use in clinical medicine is a very important problem Although our chemists are doing an excellent job, I wonder if any thought has been given to the possibility of getting tumours of the pituitary which produce excessive amounts of hormones to grow in tissue culture and thus provide a source for the production of hormones Some attempts have been made along this line but I do not know if they have been successful

Anderson I should just like to say how much I have enjoyed and benefited from the privilege of attending this colloquium

Russell One central problem has come up here as it always does with any hormone and that is what is the normal function of the hormone? With the tropic hormones of the pituitary this is easy to demonstrate in human subjects as well as in animals With respect to growth hormone or to other metabolic factors of the pituitary however we have very little information as to what these hormones actually do in for example normal adult men We know of course that growth hormone is necessary for growth in the young individual but does growth hormone have a function in the normal adult animal? Do the metabolic factors have any functions in the normal animal of any age?

Atria I would only say that I am glad to have had the opportunity to learn so much at this colloquium about the pituitary field in which we are interested in Santiago

Sonenberg Although there has been a good deal of agreement about many of the biological and biochemical effects I have noticed at times that almost opposite effects were observed by different experimenters One point that for me deserves re emphasis is that al though we constantly think of the homeostatic mechanisms in terms of the pituitary in a target gland I should like to remind myself that there are other homeostatic mechanisms which are operative in some of the end points that we are measuring This is most apparent with blood glucose of course but some of the other end points that we measure are similarly controlled by other factors

Young I agree with Dr Jane Russell that the normal function of human pituitary hormones is an important goal at which we are aiming but it is clear too that we shall achieve that goal by knowing more about functions of hormones which are more easily available at the present time than human pituitary hormones although human pituitary hormones will obviously contribute to that end We need also to know much more about normal metabolic processes and the influence of hormones on these processes in the human being It is only relatively recently that we have begun to take biopsy specimens and so to investigate metabolic processes in human tissues and the possible influence of hormones on these processes

I am surprised that in our meeting we have not heard more about chemical and biological criteria of purity of the materials we have been talking about This is always an important point with respect to materials prepared from natural sources and particularly with respect to proteins and peptides prepared in this way Though an overlap of properties appears to exist between certain protein hormones we still are not certain in many instances whether the materials prepared from natural sources are single entities Certainly we wish to learn more about the possible methods for purifying FSH ICSH and TSH and also possibly prolactin Already growth hor mone is being dealt with very skilfully in the hands of Prof Li and his colleagues and we know more about corticotropin as a chemical substance than about the other anterior pituitary hormones that we have been discussing Those of the pars intermedia which were con sidered this morning have also been purified and structurally analysed with the application of chemical criteria of purity in a way that is not possible with others Unfortunately the function of the MSH s in the human being is still a matter for discussion although if he were here Dr Lerner could tell us about an important influence of MSH on pigment production in skin including human skin The

factors related to the problem of hypertension and of compensatory hypertrophy of the kidney Testicular and thyroid hormones can modify renal growth (Braun Menendez E (1958) *Ann intern Med* 49 717) and adrenal hormones are related to at least some forms of arterial hypertension (Braun Menendez, E Fasciolo J C, Leloir L F, Muñoz J M and Taquini, A C (1943) Hipertension arterial nefrogena Buenos Aires El Ateneo) In heart failure secondary hyperaldosteronism and increased release of alcohol dehydrogenase (Leiter L (1955) *Bull St Francis Hosp (Roslyn)* 12 1) greatly handicap the natural progress of many patients and pose practical problems worth studying Finally rheumatic fever and collagen diseases are somewhat related to pituitary and adrenal cortical hormones and clinical benefit ensues from their administration to countless patients

These are but some of the problems which require clarification The field is wide open and I hope that we may have some answers in the near future

Gómez Mont I should like to comment on some of our work on debilitated patients in whom we have studied the urinary gonadotropin excretion with reference to what Dr Loraine has said earlier We have found that young undernourished women have a low gonadotropin excretion when they have potentially normal ovaries Postmenopausal women usually have a very large excretion of urinary pituitary gonadotropins during the acute stage of malnutrition

We have done the same type of studies on debilitated patients with cancer of the cervix We have found that in postmenopausal patients bearing this type of tumour, the urinary gonadotropin excretion is very low We find at the same time a large number of cornified cells in the vaginal smear and urinary excretion of oestrogens These patients have been submitted to ovariectomy and occasionally we have observed an increase in the urinary gonadotropin excretion after the operation A few of these castrated patients have been submitted to bilateral adrenalectomy and as a rule they show a marked increase in urinary gonadotropins after the operation These findings bring me to two important points first there may be an adrenal factor that can suppress urinary gonadotropins in patients with cervical cancer the second point is that a similar factor seems to be responsible for suppressing the urinary gonadotropin excretion in young undernourished females This factor can be of ovarian or adrenal origin Our findings do not support the hypothesis that this low urinary excretion of gonadotropic hormones in malnutrition is due to a primary pituitary deficiency in the elaboration of gonadotropic hormones

including man and the species variations in their structures a new light may well be thrown on biological evolution This is probably a matter for long term consideration in the future

These are some of the general considerations that I have borne in mind during the course of this meeting No doubt there are many other lines for development which you may care to suggest

Luft Prof Young to bring up the question of the tools that are available in metabolic research in humans there has been some development in this respect during the last ten years During the nineteen forties metabolic study was confined to intake and output of different compounds and calculation of the net balance In the beginning of the nineteen fifties isotopes were introduced into metabolic studies mainly isotopes of the electrolytes sodium potassium and bromide which made it possible for us to study shifts in the electrolyte content in different body compartments During this colloquium we have heard that we can include in our studies estimations of metabolites of glucose and fat in the peripheral blood But I agree completely with Dr Russell when she says that what we measure is some kind of sum total of what happens in the body and that such data are very difficult to interpret

I would like to add another tool for metabolic research in the human to the ones already mentioned For some special reasons we decided to study metabolic processes in human muscular tissue A technique was developed by Dr Ernster Dr Ikkos and myself for this purpose Biopsies were taken from muscles in humans during operations under general anaesthesia and under local anaesthesia The tissue was homogenized and the mitochondria isolated and washed The respiration of these mitochondria was then studied The subjects studied were one group of patients operated on for non metabolic disorders and three hypermetabolic patients Of the latter group one had a classical thyrotoxicosis one a thyrotoxicosis factitia and one a severe hypermetabolic state of non thyroid origin What we found may be briefly summarized as follows There were consistent differences between the two groups with respect to the extent of respiratory control As expressed in terms of stimulation of respiration by hexokinase and glucose the respiratory control was much higher in the normal than in the hypermetabolic mitochondria Similarly in the case of the adenosine triphosphatase (ATPase) activity a mean stimulation by dinitrophenol of about three to fourfold was obtained in all normal cases whereas the stimulation was threefold in one hypermetabolic case and less than twofold in the remaining three experiments In addition normal phosphate/oxygen ratios could be obtained in mitochondria with a markedly lowered respiratory control

function of prolactin in the human being and particularly in the adult male is a matter which we have not touched upon at all I would like to know, and I suppose we shall not know for some time whether human prolactin is luteotropic in the human being

We shall have to consider in the near future and are already considering in some instances the identification of human pituitary hormones in body fluids their isolation from tissues and body fluids their assay in blood and other fluids and the possible pathological existence of abnormal forms of these hormones All this is clearly ripe for fruitful development In that connexion we need to develop methods of biological assay which are satisfactory for human pituitary hormones and for this purpose I wonder whether at some stage we shall not have to consider the setting up of international standards of hormones from the human pituitary gland since biological comparisons with hormone standards of different species may well be misleading

Dr de Ulhoa Cintra emphasized the value of immunological studies in hormone investigation and I certainly support that view myself particularly with respect to hormone assay and in the investigation of hormones and their relations in body fluids and tissues generally

We have not discussed very much although we have touched upon it the relationship between placental hormones and pituitary hormones The placenta is a tissue easily available in large amount and possibly of value in yielding hormone active materials which warrant investigation As I suggested in my opening address we are now within sight of being able to study the pharmacology of some pituitary hormones the effects of variation in chemical structure on biological activity a subject on which Prof Li touched During the next ten years we can confidently expect the production of many non naturally occurring peptide hormone structures the biological activity and possible usefulness of which will be of great interest

Another subject for further investigation is the inhibition of human pituitary hormones by antagonists artificial or natural investigations on the control of their secretion may well relate to this Concerning the mechanisms of action of human pituitary hormones investigations *in vitro* and *in vivo* will both be extremely important We shall ultimately wish to know much more about the processes involved in the biosynthesis of human pituitary hormones and how it is that the human pituitary gland can apparently produce a large amount of protein hormone as it is needed particularly growth hormone The catabolism—the breaking down of these pituitary hormones in the human body—is also clearly a matter for further investigation

By the time we know more about hormones in different species

There seems to be a second area where the pathway may be upset. These studies are only very preliminary and I mention them simply because in Dr Luft's hands in the normal he showed very little change in these parameters whereas I think that if you study it in the hypopituitary patient or in the diabetic you may well be able to demonstrate changes

Wilhelmi Prof Young spoke of the possibility of exploiting the placenta as a source of pituitary hormones or pituitary hormone analogues We had this notion some years ago and made an attempt to extract human placenta by the original procedure which had been used for pituitary growth hormone As things went along it seemed very promising because at the point at which we obtained very nice crystals from the pituitary we also obtained a very nice crystalline preparation from the placenta This preparation was completely inactive as a growth promoting substance We have not taken this problem up again but in the light of our experience with other methods and with human tissues I think perhaps we might resume the study of the placenta as a possible source of growth hormone Incidentally it may be possible that since the placenta is at a region in which an intensely growing individual is growing within another individual there may be a need for regulation of growth at this site so that the substance which we precipitated which was not growth promoting might well be worth studying from the standpoint of whether it could be growth inhibiting This is another notion which we hope to pursue in the near future

Young My colleagues at Cambridge and I have also made attempts over the last few years to prepare growth hormone active material from placenta The results were not so encouraging as we had hoped We believe that there is some activity in placenta but it is very difficult to purify

Wilhelmi One can make a very pessimistic calculation the human pituitary is about 500 mg and a good human placenta is about 500 g If the same quantity of growth hormone is all that is needed to be kept in reserve by the placenta then instead of having say 5 or 6 mg per pituitary we have 5 or 6 mg /0 5 kg of placenta and a very difficult problem of isolation

Young Nevertheless the availability of placental material on a large scale leads me to hope that ultimately purification will be possible

Wilhelmi If we can work out an efficient method of disposing of the residue!

Li Prof Wilhelmi I am wondering about your mention of the growth promoting activity of the fraction that is not active in the rat I have been quite interested in this problem. I feel that one

It was suggested by Lardy that the respiratory control of the mitochondria *may constitute the biochemical basis* of thyroid hormone action This effect has by some authors been interpreted as a secondary consequence of a lowered phosphorylative efficiency, a deficient complement of bound co factors (diphosphopyridine nucleotide) or swollen mitochondria Such explanations have been eliminated by our studies

I have mentioned this study because it demonstrates that it may be possible to use human tissue for metabolic studies and that it may be possible to study metabolic processes at the subcellular level in humans We are now extending our enzymic studies on human muscle to include the supernatant fraction after centrifugation of the homogenate

Young This is indeed a pioneer study and blazes a trail for much future research

Beck Drs Dorothy and Philip Henneman (1959 personal communication) made some observations on intermediary carbohydrate metabolism in panhypopituitary dwarfs receiving growth hormone and as I recall they were able to show that immediately after the first injection of growth hormone there was a prompt and simultaneous rise in plasma unesterified fatty acid urinary acetone total ketones and serum lactate or pyruvate This would suggest rapid fat mobilization the conversion of two carbon atom fragments to citrate and their subsequent utilization They were also able to show in hypopituitary subjects that with continued administration of growth hormone the rate of disposal of the two carbon atom fragments seemed to be much improved because the urinary and plasma ketones and acetone fell and although the unesterified fatty acid levels declined they did not do so nearly as strikingly From about the 5th to the 7th day of growth hormone administration the α ketoglutarate became markedly elevated On the basis of these observations Dr Dorothy Henneman partly in Boston and subsequently in our laboratory in Montreal has been studying the intermediary metabolites of the tricarboxylic acid cycle in mature onset diabetes and our data so far fall into two groups (1) Those individuals with mature onset diabetes who do not get nitrogen retention with growth hormone and in which you can find evidence of inhibition of glycolysis of very marked fat mobilization and also an increased production of tricarboxylic acid metabolites (2) Those with mature onset diabetes that do get anabolism you can show evidence of depression of glycolysis and of fat mobilization by the same criteria Interestingly enough the elevation in the α keto glutarate and in citrate which fails to occur in the diabetic who does not show an anabolic effect occurs in the diabetic who does

various methods for showing growth hormone activity in the placenta both at home and in Prof Li s laboratory two years ago It was possible to show that there was activity in some at least but I would suggest that the amounts were extremely low Furthermore the placenta is one of the worst organs to fractionate because when animals are used for assay and the preparations are not pure enough the animals get sick and cannot be used as test animals

Wilhelmi Another topic which arises out of this colloquium is that the discoveries that we have made about the human pituitary proteins throw a deeper light on the necessity for studying more intensively those proteins that are readily available At present we are like discoverers around an island we know something about the shores of the island but nothing of the interior and it seems to me that the solution of our present problem of plentiful supply will be ultimately the complete chemical definition of the non primate hormones and the study of the modifications necessary to make them effective in human subjects Don t you agree Prof Li?

Li This problem has interested me for years We have tried to modify beef growth hormone with chymotrypsin and there is some indication that one of the active fragments after digestion with chymotrypsin is active in man The clinical experiments were carried out by Dr Peter Forsham and co workers in San Francisco (Forsham P H Li C H DiRaimondo V C Kolb F Mitchell D and Newman S (1958) *Metabolism* 7 762)

Raben Two points related to immunological considerations Prof Russell Fraser asked Dr Read whether any other preparation of growth hormone was satisfactory for the immunological procedure he had used for measuring growth hormone in blood Li and Hayashida have published that Li s beef growth hormone produces antibodies to bovine globulin and bovine prolactin as well as to the growth hormone (Li C H and Hayashida T (1958) *Endocrinology* 63 487) I wondered whether his human preparation does that in which case it would be impossible to use it for this purpose

The other immunological point that I was curious about was mentioned by Dr Gemzell i e that animal FSH given to human subjects produces antibodies which then react with human FSH It is an important point for future work on assays to inquire whether this has been rigorously demonstrated

Gemzell The work I referred to was done in Copenhagen by Oestergaard He was able to show that various animal preparations (I think he was mainly working with pregnant mare serum) of gonadotropin could produce antihormones in man and that these antihormones could actually be isolated and when injected together

must look into the activity of a fraction in other animals as well as just in rats or mice To me this is one of the endocrinological problems that look very exciting As I mentioned already for instance a mammalian ACTH is active *in vitro* in stimulating the adrenal gland to produce aldosterone (Carstensen H Burgers A C J and Li C H (1959) *J Amer chem Soc* 81 4109) We have also observed that beef growth hormone is active *in vitro* as the ovulating agent in the tree frog *Rana pipiens* (Burgers A C J and Li C H (1960) *Endocrinology* in press) When fragments of the ovaries of the tree frog were soaked for 24 hours at room temperature in a hormonal solution in a concentration of as low as 50 µg /ml of Holtfreter s solution the growth hormone was found to be quite potent in inducing ovulation Thus I wonder whether the fractions from the placental tissue should be tested in other species It might be very interesting

Young That is a good point Prof Li the problem of the species one chooses for biological tests is of great importance To purify something active in the human being I would naturally choose not the tree frog but the rat I imagine that one would try to go up the evolutionary scale rather than down

Wilhelmi Thank you very much for the suggestion Prof Li Since I have run into the question of specificity, it should have occurred to me I would like to add a little to the story of specificity Dr Grace E Pickford who has been collaborating with me in the work on fish pituitaries and the response of fishes to various growth hormone preparations has now had the opportunity to study the effect on growth of hypophysectomized fundulus not only of bovine growth hormone but also of sheep monkey and human as well as fish growth hormone Sheep growth hormone is as effective as bovine growth hormone very active Monkey and human growth hormone are only about one sixth to one eighth as active although in the rat they are at least as potent as the sheep preparation So now we have the fish responding to its own species of growth hormone to sheep to bovine and very inefficiently to monkey and human growth hormone Whether this has some relationship to the slight trace of cross reactivity which Prof Read has observed between fish growth hormone and the human antibody remains to be looked into further

Gemzell I have been working on the growth hormone content in the placenta for many years and my colleagues and I were able to show five years ago that the placental blood was high in growth hormone activity and at the same time the peripheral blood of the woman was low or not measurable (Gemzell C A Heijkenskjold F and Strom L (1955) *J clin Endocr* 15, 537) I have tried

is the biosynthesis of pituitary polypeptide hormones It would obviously be difficult to undertake studies of this kind in the intact animal and I should like to know if it is possible to grow pituitary cells in tissue culture and if so whether one could keep on producing hormones long enough to obtain quantities which would be sufficient for the application of chemical methods to the investigation of mechanisms of biosynthesis and to look for interrelated pathways in the biosynthesis of the various hormones which are present in the gland

Wilhelmi I can add a little to this Dr William McLimans who until recently has been at the Wistar Institute and has now come to the Communicable Diseases Centre in Atlanta has been working on this problem for some years He has a very ingenious method for culturing human pituitary cells in free culture where each cell is a single cell He has devices which when everything works well would enable him to culture cells at such a rate that he could produce perhaps 50 g of such cells per week However this is still in the pilot stage Only recently has it been possible to begin the study of the biological activities of such cultures These studies are so far incomplete At present the information I have seems to suggest that the activity which is unequivocally present is ACTH In view of the fact that there is a great deal of ACTH in the cells the question of how true the assays of other activities particularly the growth activity may have been is still a point of doubt So there is a little hope that a method of biological culture of a cell line may be worked out on a practical basis and permit the production in reasonable quantities of some of the activities So far the cells which seem to grow well in this particular line of free culture are cells which have a very nice granular character and appear to be largely acidophils there does not seem to be in the culture any of those cell types which are associated with the production of gonadotropin or TSH In the long run one may have to look out for the nurture of particular cell lines that are specializing in one or other of the active principles or the conditions of culture—which I think may be quite important—may have to be altered in order to induce the cells to change their mode and to concentrate on one or other of the hormones

Anderson We have accomplished something like that by transplanting mouse hormone producing tumours into other mice We have transplanted tumours which produce TSH and have harvested a good supply of it This has also been done with ACTH producing tumours

Raben I have had the opportunity of testing one line that was supposed to have produced ACTH and I could not find any biological activity

with the gonadotropins in rats inhibited the gonadotropin effect (Oestergaard E (1942) Antigonadotrophic Substances Copenhagen Munksgaard)

Sonenberg Dr Raben I have a recollection of something which is non rigorous with regard to TSH When beef TSH was administered to humans—this was originally done to stimulate the uptake in patients with thyroid carcinoma who were to be treated with ^{131}I—there was initially an increase and subsequently not only was there no increase but the uptake decreased below the initial levels This was observed by a few people and was interpreted to mean that there was cross reaction with the patient s own TSH

Raben There might be alternative explanations for that

Anderson I should like to confirm what Dr Sonenberg has just said Back in 1931–34 when we were working with TSH beef TSH produced antihormones in the rat which neutralized the rat s own TSH We demonstrated this by following the basal metabolic rate daily during continuous injections of TSH At first the basal metabolic rate rose and as we continued the injections after two weeks the metabolic rate went down and reached a point as low as that of the hypophysectomized rat On sacrificing the animals we found that the thyroid histology showed marked involutions exactly like the histology of the hypophysectomized animal (Collip J B and Anderson E M (1935) *J Amer med Ass* 104 965)

Beck Prof Li have you in the use of purified human growth hormone preparations as an antigen or in the use of your modified bovine growth hormone as an antigen ever seen cross reactivity with other anterior pituitary hormones such as you did with your unaltered bovine growth hormone antibody studies?

Li It is not cross reactive with highly purified FSH and ICSH we have not yet sufficiently pure prolactin to do this experiment

Dr Anderson could you conclude from your early experiments that you have just described that beef TSH is identical with rat TSH? We have sacrificed quite a few ml of antiserum to human growth hormone in the growing rat we thought it might be that the growing rat stopped growing after injection of antiserum to human growth hormone Unfortunately we have wasted about 50 ml of antiserum to human growth hormone and the rats are still growing Therefore we tentatively concluded that the human growth hormone is not the same as the rat growth hormone

Anderson From our study we had no right to conclude that beef TSH is identical with rat TSH but we did conclude that antibodies to beef TSH which the rat elaborated reacted with the rat s endogenous TSH

Harris A subject which to my knowledge has not been studied

ovarian response So I propose that in order to get a uniform terminology this factor should be called interstitial cell stimulating hormone rather than luteinizing hormone from now on

Loraine I entirely agree with Prof Li s suggestion on terminology At the second meeting of the Gonadotrophin Club in Birmingham in 1955 we decided that the term ICSH was preferable to LH because it referred to effects observed in both sexes I should now like to propose that the term LH like for example such terms as prolan oestrin and progestin should become of historical interest only

Anderson What about follicle stimulating hormone in the male?

Russell Fraser I would join the opposition with Dr Anderson on this theme It seems to me that until we really have a more definitive name for this than just that it stimulates the interstitial cells there is no good reason for changing from two letters to four You can if you wish call it Leydig stimulating hormone—LSH—if you find that preferable but why not call it for instance luteotropic? If we really get to a proper definition of what it is we can make a new name I feel there should always be an extremely good case for making a new name and an even better case for making a longer one out of an old name

Young I thought that Prof Li s point was that the two names exist and this situation is confusing would it not be preferable to choose one name only?

Russell Fraser I agree and I propose LH!

Loraine I think the important point is that the term LH was introduced into the field at a time when the majority of the experimental work had been conducted on female animals Now that we know that effects in males are also produced it would appear justifiable to use the term ICSH instead

Russell Fraser But surely on that analogy as Dr Anderson said you should change FSH The unfortunate thing is that ICSH was ever introduced The name LH could still be used to imply that it is interstitial cell stimulating hormone There should have been an extremely good case for changing the name many years ago I feel and there never was

Wilhelmi I think we are very fortunate You will recall that in the field of nutrition one finds many factors by using a whole range of different animals and plants and one can obtain the same substance which will activate one system do quite another thing in another system and then finally when the chemical characterization is ultimately made one acquires a chemical name for the substance So there they have a far more complex problem than we do I think that as far as the issue between ICSH and LH is concerned

Pearson Dr B Ray has supplied some pituitaries from acro megalic patients to Dr K Thompson of the Organon Company Dr Thompson has been working on the problem of trying to grow these tumours in tissue culture I believe that he has had some success in maintaining these cells *in vitro* but I do not know whether they pro duce hormones

Harris I had been thinking in particular of the corticotropin-MSH system The striking similarities in chemical structure which exist between corticotropins and MSH s suggest that they may be synthesized at least in part by a common biosynthetic mechan ism It is also possible that hormones are initially synthesized in the form of biologically inactive precursor molecules which are sub sequently activated in the gland by processes which are analogous to those which produce active enzymes from their zymogens Since our methods of isolation usually involve following the purification of a particular biological activity inactive precursor molecules of this type could well have escaped detection

Sonenberg Dr Leon Kraintz at the Rice Institute in Texas pub lished a thesis on the biosynthesis of ^{35}S labelled prolactin using ^{35}S methionine and characterized satisfactorily the biosynthetic prolactin by physiochemical techniques This was done with tissue slices but I do not know in what species

Young At various times during the colloquium the question of the terminology of pituitary hormones has arisen and I suggest that this is a subject we might possibly profitably spend a few minutes in discussing at the present time

Li Perhaps it is appropriate to suggest here that the terms LH and ICSH are somewhat confused in the literature Historically the first discovery of this factor was made by Hisaw and co workers in 1931 (Fevold H L Hisaw F L and Leonard S L (1931) *Amer J Physiol* 97 2) They designated it luteinizing hormone because they found it to be active in the female rats with which they worked Later the Californian group found it to be active in stimulating interstitial cells in both female and male rats (Evans H M Simp son M E and Pencharz R I (1937) *Cold Spring Harbor Symp quant Biol* 5 229) consequently the term ICSH was introduced in 1937 Since then the two terms have been interchangeable until recently when the belief has arisen among some investigators that the ICSH and LH are separate factors I think that this is unfounded they are the same substance I wonder whether this conference could standardize the terminology for this factor since now one speaker calls it LH and another ICSH I personally prefer (since I came to California) ICSH because it is appropriate for both male and female animals whereas luteinizing hormone implies only

really acts as a general metabolic hormone I would hate to call it MH although I would really prefer the term metabolic hormone for growth hormone because that would again cause confusion with mammatropic hormone Somatotropin is much easier not only for English speaking peoples but also for those speaking other European languages

Wilhelmi The growth hormone has as you say a protein character —so we could call it PH! With respect to the other metabolic hormones such as prolactin these too may not be named quite precisely We call prolactin so because it is related to lactation or at least to milk production but in the fish e g in fundulus this hormone assists in the adjustment of the fish to the freshwater environment and that is quite a different function from that which we have assigned to it by name We shall always have this difficulty until we can come to the unit of action which may be common to all manifestations of the hormonal effects As far as the gonadotropins are concerned Dr Anderson has introduced a real difficulty and I would suggest that we ought to go back to very simple terminology and call FSH G1 and ICSH G2 for the time being then there would be no possibility of confusion

Anderson May I just plead for the preservation of historical monuments for the benefit of those of us who have lived through this pituitary age and have watched first the appearance of LH on the scene and then some six years later in another laboratory that of ICSH?

Russell Fraser This is a problem that the mere physician runs into perhaps more often than the biologist i e the question of names Until we can get to the stage where we can define exactly what a disease is it might as well be Mr Smith s disease and I think we should regard the names that have been given to these hormones in exactly the same light The growth hormone was first christened growth hormone and I feel we should regard that term as quite established until it can be defined in terms of what it is chemically and the exact action it has on the cells as has been said I would suggest that if we are trying to introduce some agreement into a large number of places then we might concede a point to Prof Li over the ICSH because of the confusion which he suggests LH does introduce if he would concede the point about ignoring somatotropin

Li Mr Chairman let us consider the question of ICSH and if Prof Russell Fraser agrees we shall not discuss growth hormone

Young We should not forget the student who has to learn a number of names and letters in this connexion and if some simplification could be effected it would be of great advantage to him or her I do not think this question of nomenclature troubles the

there is one possible point of confusion which Prof Russell Fraser inadvertently brought up and that is with respect to luteotropin which would ordinarily be designated LTH in our alphabetical scheme nowadays but which occasionally is rather loosely referred to as LH This is confusing and if LH were dropped from the picture this confusion would no longer arise As far as the other confusions are concerned that is simply a matter of a quick synaptic transfer and one can recognize LH and ICSH interchangeably with no strain I am not in favour of changing the name just for this reason but we have a slight disagreement on another basis Some of us call growth hormone growth hormone and some of us call it somatotropin I still call it growth hormone because I think somatotropin says at once both too much and too little In line with Prof Russell Fraser s argument again we are faced with a hormone about whose actions we do not have a perfectly clear picture nor any definite knowledge and calling it somatotropin may be decidedly premature The non committal term growth hormone is still in spite of Dr Gross s powerful argument a better term because it leaves the whole situation open until the time comes when we can say ' this hormone does this and so let us call it something related to this precisely The same thing applies with respect to ICSH or LH

Li ICSH and LH are not new terms The latter was introduced in 1931 and the former term in 1937 and since then these two terms have been in use interchangeably It is now 1959 and this might be the proper time when as Dr Loraine has pointed out we know so much about the ICSH action in both the male and the female for us to confine ourselves to one single term in the literature—not to adopt a new term but simply to adopt one of the terms now in use for the same substance Another point of confusion as Dr Wilhelmi pointed out is the luteotropic hormone *LTH* sometimes also called lactogenic hormone or quite frequently LH in the literature Thus in order to eliminate this possible confusion between prolactin and ICSH, it would seem to be appropriate for this conference to agree upon the designation ICSH for the gonadotropin With respect to growth hormone there is another problem involved Dr Paul Weiss (1955 *In* The Hypophyseal Growth Hormone Nature and Actions p 3 eds Smith R W Jr Gaebler O H and Long C N H New York McGraw Hill) mentioned that the definition of growth is dif ficult There is no doubt that the name growth hormone is a very dramatic name but we do know also that the molecule acts not only as a growth promoting agent—promoting body weight increase or tibial or other bone growth—but that it does many other things even acting *as a gonadotropin as an ovulating factor in the frog* It also stimulates the ventral prostate and the uterus and in short

but the name of the species itself That might be the best way to start

Li Another problem in the case of growth hormone is that it is sometimes modified into another form that is still biologically active Then you need a subscript—e g for bovine growth hormone $_B$ $_B$, and $_B$—to indicate the three different types

Wilhelmi You could designate this parenthetically

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Young To conclude this colloquium I should like to say a few general words To the contributors I say—thank you very much indeed for being so clear and for keeping to your times so accurately To all the members of the colloquium I say—thank you for speaking so freely and easily and for providing such a lively discussion and to the members of the audience I say—thank you for not speaking!

members of a conference like this—we all know what the symbols mean—but there is the problem of the man who writes the textbook and the student who has to learn from it This is a serious problem but I am not sure that this conference is one that is competent to settle it Would anyone like to propose a resolution in this connexion so that we might discover if we could adopt anything by a substantial majority? Would anybody propose that we record in the proceedings of this colloquium that in the view of its members, the term ICSH should henceforth be used instead of LH with respect to one of the pituitary gonadotropins?

Loraine Yes I propose that

Li I second the proposal

Young The motion has been lost by ten votes to nine On the basis of the majority of one on this occasion do we believe that we collectively have an opinion that is worth recording?

Li I feel that ten to nine is not sufficient to overrule the minority!

Russell Fraser May I make exactly the same proposal i e that this meeting would like to record its hope that in future the hormone known as growth hormone be referred to as growth hormone rather than as somatotropin

Wilhelmi I second that proposal

Young That motion is carried by 16 votes to 6 That is a substantial majority

Wilhelmi Another question of nomenclature might be well worth considering now Prof Li and I have discussed this in the past and he has raised the point in relation to ACTH nomenclature (Li C H (1959) *Science* 129 969) This is now becoming acute with respect to growth hormones of different species of animals It would be wise if we could decide on some system of designation of the species of origin of a given growth hormone preparation It has occurred to me that one might use a subscript letter e g O for ovine B for bovine P for porcine E for equine I have used the subscript HS for human relating to *homo sapiens* but others may have a different preference!

Young In my view O stands for ox! Surely it is the animal and not the meat that comes from the animal that should be the adjective to define the gland

Li When you talk of bovine do you mean a small capital B or capital B?

Wilhelmi Subscript small capital B i e GH_B

Li A question that has always bothered me is whether you should use ovine or bovine Ovine is from sheep and bovine is from the ox but in principle I agree with you

Young There are going to be so many species to consider in time that one will have to use not a single letter in the subscript

INDEX OF AUTHORS

Plain numbers indicate a contribution by the author either in the form of a paper or to the discussions Numbers in italics indicate a reference to the author s work

SUBJECT INDEX